VITAMINS AND HORMONES

ADVANCES IN RESEARCH AND APPLICATIONS

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Preface

The Editors are pleased to present this seventeenth volume of Vitamins and Hormones

It has never been easy to delineate the boundaries of the field which Vitamins and Hormones brings into review When this publication was founded in 1943 we had in mind the general philosophy that these predominantly small molecules which function respectively as controllers of metabolism and as integrators of the many functions of the organism, have a comparable role and should be studied in comparison with one another Gowland Hopkins once referred to the vitamins as Exogenous Hormones, and the recent discoveries pointing to the control of oxidative metabolism by thyroxine and by estrogens support such a concept In plants some of the B vitamins function as growth hormones As a quite different case, thyroxine can be considered a vitamin for the cretin since it must be taken with the food indefinitely in order to allow continued normal life, other examples can be found in the realm of therapy Of course there is another reason for studying vitamins and hormones together namely their value in application In his Preface to Volume I of Vitamins and Hormones (1943), Professor E V McCollum, after comparing the functions of these substances, pointed out that "the future of preventive and of curative medicine is filled with promise in these departments of learning " To the fulfillment of this promise the shelves of our drug stores bear witness

But their very community of function and application makes more difficult the precise definition of this group of substances For the boundaries, always thin, are thinning out still more As it happens the present volume gives more than one example of this In the first place there appears here an essay on an enzyme, glucose 6 phosphatase, which in some respects has functions that would normally be ascribed to a hormone Is there anything about the definition of a hormone that would exclude an enzyme? If one of the now known protein hormones were discovered to possess enzymatic activity, it might have its name changed but it would not be any the less a hormone Serotonin the subject of another review, has many claims to be considered as a hormone for the central nervous system, but it has never quite been accepted as such perhaps because of the absence of a functional secreting gland or tissue Thus it presents a borderline case but of a different sort In Volume XV we published a discussion of another borderline substance carnitine, which is a vitamin for certain insects but otherwise is widely distributed in tissues and suspected of playing a general role in metabolism Finally, the present volume includes a comprehensive review of

yet another substance of elusive function, ergothioneine It seems likely that the immediate future will see the study of biochemical control and integration widening out to recognize and include other materials of this kind Such developments will doubtless bring up problems of much more far reaching importance than those of terminology Perhaps the biochemists and physiologists of the next generation will view these problems in the more spatial and morphological framework of interrelationships between subcellular organelles

The Editors wish to express their heartfelt thanks to the eight authors and their collaborators The virtues of comprehensiveness and critical appraisal are often so nearly incompatible that a particular debt is owed to those contributors who, like those of the present volume, can combine them without weakening either one

With this volume a change in editorship which has been impending for several years finally takes its effect Dr Kenneth Thimann of Harvard and Dr G F Marrian will become Consulting Editors, and Dr Dwight J Ingle of Chicago, with his colleague Dr Ira Wool as assistant, join Dr Robert Harris in assuming the editorial responsibilities The change will not affect editorial policy in any way

Kenneth V Thimann
Robert S Harris
Guy F Marrian

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GREGORY PINCUS

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I INTRODUCTION

1 *Nomenclature and Abbreviations*

Folic acid (FA) stands for the family of conjugated pterins and pteridines having "folic" biological activity Functionally, the folic series includes *p* aminobenzoic acid (PABA) where PABA acts primarily as part of folic acid In the conjugates, the heterocyclic nucleus is attached by a peptide bond to a *p* aminobenzoyl group pteroic acid and rhizopterin are the only nutritionally active members of the heterocycle containing members of the folic series which do not contain at least one glutamyl group Pterins are aromatic, pteridines are ring hydrogenated and so have fewer double bonds Accordingly, pteroyl L glutamic acid is a pterin and tetrahydrofolic acid, a pteridine

The following abbreviations are used pteroyl L glutamic acid, PGA, di hydrofolic acid (7,8 dihydro PGA), FH_2, tetrahydrofolic acid (5,6,7,8 tetrahydro PGA), FH_4 *Citrovorum factor* (CF) *can mean a particular com pound* N^5 *formyl* FH_4 *(also called folinic acid),* usually employed as the synthetic leucovorin, or an activity for *Pediococcus cerevisiae* (*Leuconostoc citrovorum*), depending on context Distinction likewise is drawn between activity for *Crithidia fasciculata* and particular *Crithidia* active compounds, e g biopterin (Section VIII, 3) A one carbon unit in group transfer is de noted as C_1 Formiminoglycine is FIG, formiminoglutamic acid, FGA, adenosine triphosphate, ATP, disphospho- and triphosphopyridine nucleo tides, DPN and TPN, coenzyme A, CoA

An inclusive name is needed for conjugated and unconjugated pterins and pteridines since these terms are incrusted with imprecise usages and, as brought out here, there may be different functions for compounds act ing at the pterin oxidation level and for those like folic acting primarily as conjugated pteridines *Pterinoid* is proposed here, by analogy with steroid

and carotenoid, for compounds with the pterin ring skeleton Figure 1 shows tetrahydrofolic acid

2 *Significance of the Pterinoids*

Pterinoid investigations have steadily extended knowledge of (1) transfer of C_1 groups, (2) biosynthetic and functional connections between folic vitamins and other nitrogenous heterocycles—purines, thymine (or thymidine), riboflavin, vitamins B_6 and B_{12}, and unconjugated pterins

Ever since F G Hopkins looked into the pigments of butterfly wings, these unconjugated pterins (e g xanthopterin and leucopterin) have been a biosynthetic enigma The advent of folic acid complicated matters A solution is in sight thanks to other lines of investigation converging (1) the eye pigments of *Drosophila*—classical objects of genetics—are predominantly pterins in some mutants, (2) the flagellate *Crithidia fasciculata*

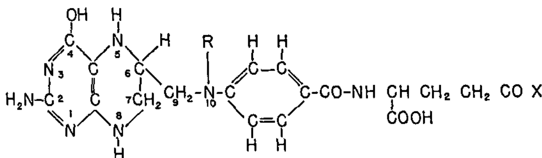


FIG 1 Tetrahydrofolic acid (FH_4) X = 1 or more glutamyl groups

requires certain unconjugated pterins, PGA apparently being the precursor (3) remarkably high concentrations of pterins, some with *Crithidia* factor activity, occur in blue green algae and are somehow involved in photosynthesis Evidently, certain unconjugated pterins have an essential, unknown function

More knowledge of pterinoids means renewed hope of solving such old clinical problems as the pathogenesis of sprue Increased interest too, attaches to the unconjugated pterins in the body Are they increased or diminished in some diseases? The effectiveness of antifolics in the palliative treatment of leukemia is halted by toxic symptoms and, eventually by drug fastness, this emphasizes the need to explore pteridine metabolism to find new opportunities for chemotherapy aimed at pterinoid targets

This review attempts to collate clues to new relationships and functions of pterinoids, its point of departure is the exhaustive treatment of PABA by Wright and Taormina (1954), and of the folic vitamin family by Stokstad *et al* (1954) Uses of microbial systems" and the comparative biochemistry of pterinoids will be emphasized The well established folic

mediated reactions and their connections to other metabolic sequences are adequately summarized in Mehler's (1957) and Fruton and Simmonds' (1958) textbooks

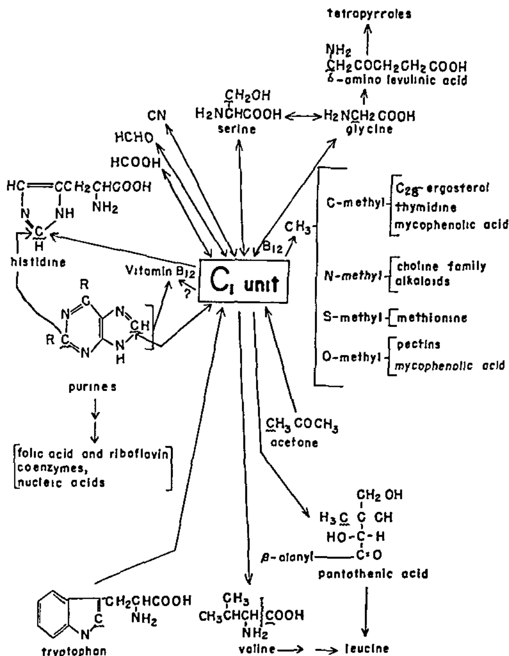


Fig 2 Some biosynthetic relations of C_1 units Adapted and enlarged from Fruton and Simmonds (1958)

Arnstein (1057) has reviewed C_1 reactions, and Greenberg and Jaenicke (1957) the biosynthetic activities of folic acid The stability of reduced pterins is discussed by Blakley (1958) The defined folic enzymes are clas

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II. Enzymes Interconverting PGA Derivatives

1. *Pterin Reductase*

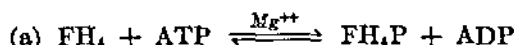
A preparation (Wright and Anderson, 1958) from *Clostridium sticklandii* catalyzed the reaction PGA + pyruvate + CoA → acetyl CoA + FH_2. Since the diglutamyl and triglutamyl pterins and their N^{10} formyl derivatives were reduced, such a reductase system can account for the transformation of exogenous PGA into the reduced coenzymatic products. An enzyme from *Micrococcus lactilyticus* formed FH_2; the reaction required an electron donor such as pyruvate or α ketobutyrate, a dithiol such as dimercaptopropanol, orthophosphate, and reduced CoA. This enzyme did not form FH_4 derivatives.

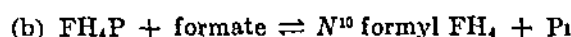
2. *Dihydrofolic Reductase*

A preparation from chicken liver (Osborn and Huennekens, 1958) carries out the reduction FH_2 + 2TPNH → FH_4 + 2TPN. Peters and Greenberg (1958a) briefly reported an acetone powder preparation of FH_2 reductase that worked best with DPN < pH 5.5 and with TPN > pH 6.0, the combination DPN + TPN did not increase reduction, suggesting one enzyme. A preparation from avian liver was inhibited noncompetitively by extremely low concentrations of aminopterin and amethopterin (Osborn *et al.*, 1958a). The authors suppose the reduction steps to be the most sensitive site of inhibition by these antifolics—an idea according with results with other systems (Section II, 2).

3. *FH_4 Formylase*

According to Jaenicke (1958), who prepared the enzyme from pigeon liver, formylation proceeds in two steps:


(a) $FH_4 + ATP \xrightleftharpoons{Mg^{++}} FH_4P + ADP$


(b) FH_4P + formate ⇌ N^{10} formyl FH_4 + Pi

Phosphorylation + reduction gives an acid unstable intermediate. As

mediated reactions and their connections to other metabolic sequences are adequately summarized in Mahler's (1957) and Fruton and Simmonds' (1958) textbooks

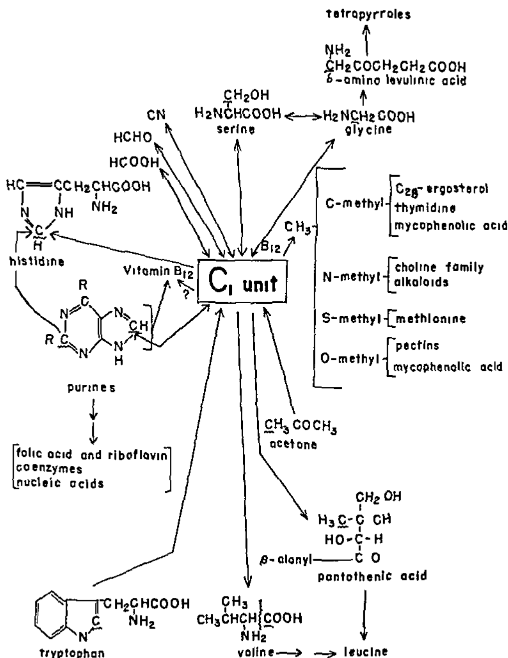


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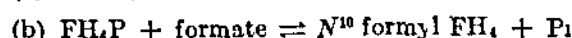
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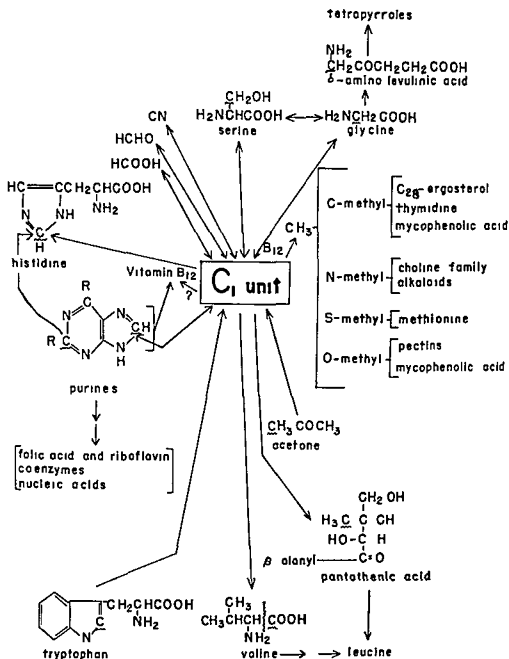


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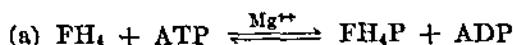
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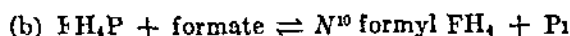
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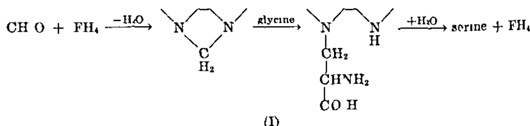
noted in the discussion of folic catalyzed degradations, deformylation is an important ATP generating reaction for some bacteria

4 Hydroxymethyl FH_4 Dehydrogenase and Formaldehyde-Activating Enzyme

Nonenzymatically, HCHO reacts readily with FH_4 at neutral pH and at room temperature to form one or more products active in systems requiring hydroxymethyl FH_4, this does not tell whether "active formaldehyde" is attached at N^3, N^5, N^8, or N^{10} In the glycine ↔ serine system, HCHO must be activated enzymatically for the reaction to proceed (Osborn and Huennekens, 1957 Osborn *et al*, 1958b)

Formation of a $N^{5\,10}$ methenyl bridge (in other terms, an imidazolidine ring) by removal of water accounts for the stability of the enzymatic hydroxymethyl FH_4 product, the synthetic compound is sometimes called anhydroleucovorin

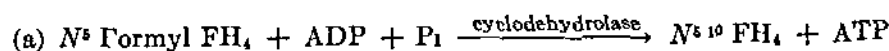
As discussed by several reviewers (notably, Huennekens *et al*, 1958, Coon and Robinson 1958), folic coenzymes at the hydroxymethyl and formyl oxidation states are almost certainly interconverted by way of $N^{5\,10}$ FH_4 bridge compounds Kisliuk (1957) proposed an attachment of active formaldehyde to the methylene carbon in the glycine ↔ serine conversion (I)



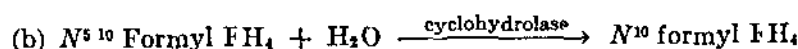
Kisliuk has suggested further that there may be a dismutation between two molecules of FH_2 to yield FH_4 and PGA

5 N^5 Formyl FH_4 Isomerase (Cyclodehydrase + Cyclohydrolase)

This enzyme provides a means of converting CF to "active formate" From recent studies, including unpublished work referred to by Huennekens *et al* (1958), the interconversion is thought to proceed via the bridge compounds



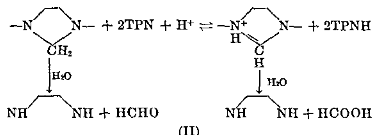
(a) N^5 Formyl FH_4 + ADP + P_i $\xrightarrow{\text{cyclodehydrolase}}$ $N^{5\,10}$ FH_4 + ATP



(b) $N^{5\,10}$ Formyl FH_4 + H_2O $\xrightarrow{\text{cyclohydrolase}}$ N^{10} formyl FH_4

Gunsalus and Sagers (1958) propose a methylene methenyl conversion at the bridge as the actual site of reduction The reaction might be formu

lated as in (II)

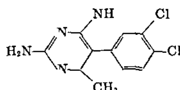


(II)

Peters and Greenberg (1958b) made from liver an acetone powder preparation of cyclodehydrolase that yielded a product with an absorption spectrum similar but not identical to $N^{5,10}$ methenyl FH_4 It has an absorption spectrum at 343 mμ instead of 354 mμ, was stable at pH 6 8, and readily autoxidizable

6 Action of Antifolics at the Reductase Steps

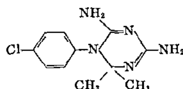
Identification of dihydrofolic reductase as the main site for action of aminopterin and amethopterin (Section II 2) fits with observations that in many systems these antifolics are more efficiently reversed by CF than by PGA, e g chick embryos (Naber *et al*, 1952) weanling rats (Sauberlich, 1953) *Lactobacillus arabinosus* (Hendlin *et al*, 1953) tissue cultures (Jacobson 1954) also amethopterin resistant *Streptococcus* related PGA derivatives (Broquist *et al*, 1953) Burchenal (1956), in reviewing folic acid antagonists in cancer therapy notes that the 4 hydroxy derivatives of PGA are reversed by PGA rather than by CF while diaminodichlorophenyl pyrimidines (e g formula III) seem to compete even more with CF than



2 4 Diamino-5 (3 4 dichlorophenyl) 6 methylpyrimidine

(III)

do the 4 amino PGA's such as aminopterin and amethopterin Dihydro triazines such as (IV) act as noncompetitive inhibitors of PGA and CF in



4 6 Diamino 1 (*p* chlorophenyl) 1 2-dihydro 2,2-dimethyl s triazine

(IV)

Streptococcus faecalis or *Pediococcus cerevisiae* (Foley *et al*, 1955) Only the 4 amino PGA derivatives seem to have a selective effect on leukemia in children, other leukemias, such as the myeloid mouse leukemias, are resistant Resistance to one antifolic, e g amethopterin, generally confers resistance to all other folic antagonists, including the diaminodichlorophenylpyrimidines

Folic antagonists will be mentioned elsewhere in connection with folic splitting enzymes (Section VII) and purine synthesis (Section IV, 1)

7 *Comparative Biochemistry and Pterin Reductases*

A unique feature of folic mediated group transfers is that a change in oxidation level of the group may accompany some transfers

As seen, pteridines, not pterins, mediate C_1 transfers, PGA is, enzymatically, an artifact This situation recalls the prevailing idea that anaerobic systems are the primitive ones Pteridine dependent organisms confronted with atmospheric oxygen originating from the photosynthetic activities of green plants (the bacterial photosyntheses are anaerobic) presumably developed PGA reductases or perished The acidity of the gastric contents converts CF to PGA (Hausmann and Mulli, 1952) This, coupled with the extreme autoxidizability of FH_4 derivatives, provides a teleological argument for the evolution of reductases to ensure the utilization of so convenient a stabilization product as PGA It should be recalled, too, that the adduct of HCHO and PGA, which is stable toward bases, releases HCHO in acid (Blakley, 1957 a,b) So far, all metazoans studied carefully have a folic requirement satisfied by PGA No photosynthetic organisms are known which require exogenous FA or riboflavin, it may be significant that these vitamins are much less light sensitive in the reduced or leuco forms Fastidious FA requiring parasites with poor reducing powers would be expected to prefer FH_4 CF compounds to PGA The Reiter treponeme, an anaerobe, requires CF PGA is inert (Steinman *et al*, 1954) The CF requirement of *Pediococcus cerevisiae* seems much less rigorously determined, probably reflecting minor defects in reducing and formylating ability 27 of 34 strains were satisfied by PGA as well as by CF (Jensen and Seeley, 1954) Moreover, measures that enhance the reducing powers of the culture medium, e g addition of ascorbic acid, or of the organism—as by adding CoA (Foley *et al*, 1955)—enhance the utilization of PGA or diminish the effect of antifolics such as amethopterin An intensely aerobic, fastidious parasite such as the malaria organism (Trager, 1958) may prefer CF to PGA Such a parasite presumably has intense reducing ability, perhaps some of its fastidiousness may come from its having to be supplied with the nutrients required to organize a reducing system

III Folic Coenzymes in Catabolism

1 Generalities

A vitamin may participate in synthesizing cellular catalysts and in degrading major substrates Assuming that the turnover numbers—the wear and tear—of the vitamin as well as the enzyme affinities are about the same for both activities, then a major substrate reaction should demand much more folic enzyme than would the syntheses of quantitatively minor cell constituents Substrates dissimilated anaerobically would be especially favorable for study when a C_1 unit is concerned, autoxidative destruction of folic enzymes is minimal Clostridial fermentations, as will be seen, have furthered folic studies remarkably

2 Glycine and Glutamate

Paretsky and Werkman (1950) found an *Achromobacter* which aerobically utilized glycine as principal substrate, HCHO appeared as an intermediate This foreshadowing of the involvement of folic acid in glycine catabolism was paralleled by work on the rat (Nakada and Weinhouse, 1953) liver homogenates oxidized glycine through two pathways (1) serine, alanine, pyruvate and the Krebs cycle, (2) glyoxylate, formate, and CO_2 Nutritional data point to FA coenzymes in some of these reactions as clearest sign, the toxicity of glycine for the chick is counteracted by PGA (reviewed by Stokstad 1954) Indeed, diets with excessive glycine and methionine (0 3–0 8%) are convenient for inducing folic deficiencies (Briggs *et al*, 1958) Serine satisfies the glycine requirement of the chick (Wixom *et al*, 1956)—an indication that the serine glycine interconversion has the capacity to be an important catabolic pathway for glycine Whether the glycine glyoxylate pathway involves folic coenzymes cannot be told in this way, for PGA did not counteract glyoxylic acid toxicity (Naber *et al*, 1956) Nevertheless, Nakada and Sund (1958) regard the glyoxylic acid pathway as being the principal degradative pathway for glycine in the liver and as folic mediated They purified an enzyme system from liver mitochondria which oxidatively decarboxylated glyoxylate The reaction required DPN, thiamine pyrophosphate Mn^{++} or Mg^{++} and, almost absolutely, glutamic acid *N* Formylglutamic acid was isolated as an intermediate They therefore postulate that the α carbon of glycine forms the β carbon of serine via a *N* formylglutamic acid intermediate which then formylates FH_4

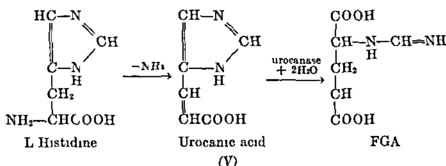
Formylglutamic acid can be formed in liver in the readily reversible reaction (Silverman *et al* 1958b) 5 formyl FH_4 + glutamic acid $\rightleftharpoons$ FH_4 *N*-formylglutamic acid a reaction also observed by Miller and Waelsch (1957)

Glycine degradation by bovine spermatozoa is thought to go through glyoxylate, formate, and CO (Flipse and Benson, 1957) A folic coenzyme, if required here, should be extraordinarily interesting Does the sperma tozoon retain the folic coenzyme used in the synthesis of its DNA?

Bacteria can make formate in many ways A preliminary note by Chin *et al* (1957) reports that the "phosphoroclastic" splitting of pyruvate to $HCOOH + CH_3COOH$ by *Escherichia coli* was stimulated by FH_4 Huen nekens *et al* (1958) refer to similar unpublished results (Whitely, 1958) with *Micrococcus aerogenes* and *M lactilyticus*, serine was not an inter mediate If the reaction $HCOOH \rightleftharpoons H_2 + CO_2$, also carried out by *E coli*, turns out to be folic catalyzed, the presumption would be greatly strength ened that all formate CO reactions require FA, as might perhaps also for mate oxalate reactions

3 Histidine Dissimilation and Formimino L Glutamic Acid

Formiminoglutamic acid (FGA) accumulates in the urine of folic deficient animals because of the blocking of the FH_4 mediated reaction needed for its degradation (V)



L Histidine Urocanic acid FGA

(V)

The formimino group is then transferred to FH_4, as with formimino glycine (Section III, 4), with eventual liberation of formate ammonia, and glutamic acid *Aerobacter aerogenes* converts FGA to L glutamic acid and formamide, the formamide then going to NH_3 and CO , *Pseudomonas fluorescens* forms *N* formylglutamic acid as an intermediate (Magasanik and Bowser, 1955) The anaerobic breakdown of histidine apparently in volves the same intermediates formamide accumulates in cultures of *Clostridium tetanomorphum* fermenting histidine (Wachsman and Barker, 1955) This strain also ferments urocanic acid and glutamate, the labeling of the formamide is consonant with the aerobic sequence [Curiously, formamide is a weak carcinogen *E coli* strains resistant to the weakly carcinogenetic urethan were cross resistant to urethan and *N* methylformamide, for refer ences, see Skipper and Bennett, (1958)]

The aerobic degradation of histidine by an intensely aerobic *Pseudomonas* isolated by the enrichment technique has revealed another pathway—one

with N formimino and N formylaspartate as intermediates (Hayaishi et al, 1957, Ohmura and Hayaishi, 1957)

The urinary excretion of FGA has been used as an index of folic, i e FH_4, deficiency Tabor and Wyngarden (1958) find that the microbiological method, based on the release of glutamic acid from the microbiologically inactive FGA on heating was complicated by the glutamic acid and glutamine normally present in urine and by the variable conversion of these to pyrrolidone carboxylic acid during the heating step They propose, instead a spectrophotometric assay based on the formation of $N^{5,10}$ methenyl FH_4 which absorbs strongly at 350 mμ (see Section II, 5) Urine is incubated with FH_4, purified extracts of acetone powders of hog liver containing FGA transferase, which (a) forms N^5 formimino FH_4, and formimino FH_4-cyclodeaminase which (b) cyclizes the product to $N^{5,10}$ methenyl FH_4 As a control the FGA is destroyed by incubating at pH 11 5 at 25° for 4 hours Silverman et al (1958a) propose a method based on the formation of N^{10} formyl FH_4 from FGA and PGA in a chicken liver homogenate also containing PGA reductases and cyclohydrolase, in this they then measure CF activity microbiologically

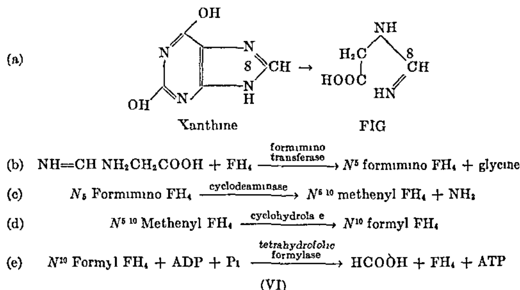
As a check on these methods for studying folic deficiency states, Rabinowitz and Tabor (1958) measured the increased urinary formate in folic-deficient rats, the formate was not derived from histidine The formate was converted by FH_4 formylase + ATP to N^{10} formyl FH_4, which was then converted by acid to $N^{5,10}$ methenyl FH_4 The formic acid was thought to originate from a variety of sources which did not need folic cofactors, formic acid then accumulated in the absence of the folic cofactors for metabolizing it further Tryptophan was one such nonfolic source of formic acid its administration increased urinary formic acid, especially in folic deficient rats Silverman and Pitney (1958) have shown that the excretion of FGA was significantly reduced or eliminated by methionine or B_{12} even with extra dietary histidine Connections between FA and B_{12} are discussed below (Section V) That folic acid may play an additional role in histidine degradation in higher animals is indicated by the report that liver urocanase was lowered in folic deficient rats (Baldridge, 1958)

Recent studies are illuminating the manifold cross connections between the metabolism of formyl or hydroxymethyl and methyl groups A direct route connecting formaldehyde and the methyls of choline bypassing the long established B_{12}-catalyzed serine β carbon + homocysteine → methionine route (Section V, 2), is rendered probable by the Venkataraman and Greenberg (1958) findings, these workers prepared a soluble system from liver which synthesized choline methyl groups from HCHO with either ethanolamine or diethanolamine as acceptors and FH_4 the cofactor No choline was here synthesized from methionine It will be interesting to see

whether any of these enzymes require B_{12} as cofactor as the serine homo cysteine route (Section V, 2) does

4 Purines and Formiminoglycine

After discovering that extracts of *Clostridium cylindrosporum* and *C acidiurici* which can derive their N, C, and energy from purine, e g xan thine, or 4 aminoimidazole, accumulated formate among other products, Rabinowitz and Pricer (1956) identified formiminoglycine (FIG) as an in termediate, and then showed that formimino group transfer was involved Details have since been adduced (VI, a–e) (Rabinowitz, 1958)



The enzymes catalyzing each reaction were purified, FH_4 formylase was crystallized (Rabinowitz and Pricer, 1958) and formimino FH_4 isolated and crystallized Reaction (VI, e), as noted, is a new ATP generating reac tion

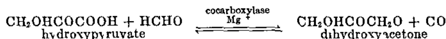
Gunsalus and Sagers (1958), in studying this sequence for *C acidiurici*, propose that dissimilation of glycine proceeds either by condensation with FH_4 and oxidation of the adduct, or else by way of serine to pyruvate and thence oxidative decarboxylation to acetate and CO_2 Formate and CO_2 are in equilibrium, thus providing another cyclic mechanism for oxidation of C_1 units A similiar reaction sequence is stated to hold for the dissimila tion of glycine by the anaerobe *Diplococcus glycinophilus* Glyoxylic acid is inert in these systems

5 Catabolism of Formate and Formaldehyde

Serine can be formed directly from a C_3 intermediate of glycolysis path way (Arnstein and Keglevic, 1956), by the amination of pyruvate, this

reaction is quite likely the most important source quantitatively of C_1 units

While formic acid accumulation in folic deficiency makes it clear that the main route for formate removal is folic mediated, little is known in this respect about formaldehyde, which arises from the oxidation of methyl groups or the folic mediated reduction of formate (Section II, 5) Perhaps an important connection between C_1 and the principal pathways for substrates is indicated by the finding that HCHO is an excellent acceptor for the ketol group ('active glycolaldehyde') formed from a variety of ketose phosphates and from hydroxypyruvate (Dickens and Williamson, 1958) This reaction may be formulated as follows



Formaldehyde also participates in aldolase reactions with dihydroxyacetone

Hydroxypyruvate can be formed from serine by transamination, dihydroxyacetone is readily phosphorylated to triose phosphate As CO_2 is removed, more hydroxypyruvate could be formed from serine Some such cycle would provide a nonfolic mediated disposition of formaldehyde Formaldehyde in turn, could be generated by the oxidation of the *N* methyl of sarcosine, sarcosine in turn is generated by the removal as "active formaldehyde" of one of the methyls of dimethylglycine (Mackenzie and Frisell 1958) Dimethylglycine is formed by the well established sequence sarcosine → glycine + HCHO → serine → ethanolamine + CO → monomethylethanolamine → dimethylethanolamine → choline → betaine → dimethylglycine

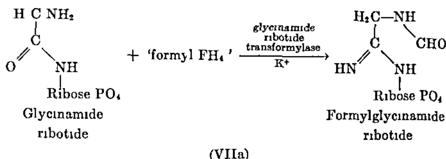
The results of McDonald (1956) showing that excess choline or glycine causes a growth depression in chicks which is alleviated by PGA imply that the nonfolic paths for disposing of excess methyl have limited capacity at least in higher animals The quantitative significance of liver formaldehyde dehydrogenase in liver a flavin enzyme with an additional glutathione cofactor (Strittmatter and Ball, 1955) is unknown

IV Some Folic mediated Syntheses

1 Purines

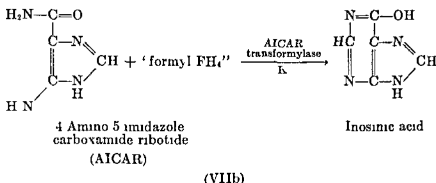
Transformylations in purine synthesis have been summarized (Warren *et al*, 1957) and purine synthesis has been reviewed (Buchanan *et al*, 1957) Purine synthesis is one of the most important uses of FA, as attested by the findings from a variety of folic requiring organisms that, in sparing a FA requirement, the efficacy of purines is almost the first to emerge This may

reflect not only the quantitative importance of purines in the economy of the cell, but also the fact that folic reactions enter at two stages (VIIa) formylation of glycinamide ribotide



(VIIa)

and (VIIb) insertion of C to form the pyrimidine ring



(VIIb)

In some systems, especially microbial ones, purine synthesis is quite sensitive to antifolics One would expect that to some extent purines would noncompetitively reverse inhibitions by antifolics Guthrie *et al* (1956) found that hypoxanthine or adenine blocks inhibition of *Bacillus subtilis* by amethopterin Also, Tomisek *et al* (1958) present evidence that formylation by AICAR is the primary site of amethopterin inhibition in *E coli*, and Ghosh *et al* (1958) found that some compounds that were competitive antagonists for PGA or CF were not at all inhibitory to *Streptococcus faecalis* when the PGA was bypassed with adenine plus thymine

Phenomena of collateral resistance probably point to multiple sites of action Two mutants of *E coli* resistant to purine antagonists were more resistant than the parent strain to amethopterin (Guthrie *et al*, 1957) In contrast in *Saccharomyces cerevisiae*, various folic antagonists, including aminopterin, were reversed by thiamine plus B_6 purines plus thymine or thymidine were ineffective (McGlohon *et al*, 1957) These authors suppose that B_1 plus B_6 participate in a pathway of purine synthesis that is less folic dependent

2 Histidine

Diverse microbiological findings pointed to a biosynthetic connection between histidine and purines, somehow involving FA They date back to Broquist and Snell's (1949) observation that *Lactobacillus casei* needed more purine when grown without histidine Later observations included (a) the PABA requirement of a yeast was spared by adenine plus methionine plus histidine (Chamberlain and Rainbow, 1954) (b) a mutant of the mold *Ophiostoma* required purine, histidine, and glycine (Wikberg and Fries, 1952), (c) certain mutants of *E coli* required either histidine or purine (Gots, 1955, Luzzati and Guthrie 1955), (d) a histidine requiring strain of *E coli* could use adenine alternately when infected with phage but not when uninfected (Burton, 1957) In the meantime identification of the formiminoglutamic stage in histidine degradation which is common also to purine breakdown (Section III 1 and 4) had raised the question of whether histidine synthesis was essentially the reverse of this degradation and, as corollary, whether purines merely contributed the formimino group A recent scheme indicates a family resemblance but not identity between the synthetic and degradative reactions The C_1 transfer step leading to imidazole glycerol phosphate, a precursor of histidine is as follows (Moyed and Magasanik, 1957) adenylic acid + ribose 5 phosphate + glutamine → histidine + 4 amino 5 imidazolecarboxamide ribotide (AICAR) This provides a means of transferring the purine C to the ureido carbon of histidine by opening the purine ring yielding the purine precursor AICAR In effect, adenylic acid acts as C_1 carrier If FA were limiting so that regeneration of the adenylic acid were impeded AICAR would accumulate The breakdown of histidine, as discussed earlier, could also furnish a C_1 unit

3 Thymine Thymidine

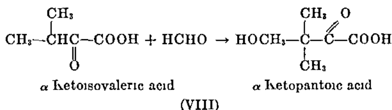
Thymine and thymidine are needed to replace folic acid for *Streptococcus faecalis*, and cells grown on thymine had no detectable folic acid activity for *Lactobacillus casei* (Stokes, 1944a b) The inference was that FA was necessary for the synthesis of thymine (or thymidine) The site of the folic reaction was soon recognized to be the 5 methyl carbon Friedkin and Kornberg (1957) found that P^{32} labeled deoxyuridylic acid was converted to labeled thymidylic acid by extracts of *Escherichia coli* As repeatedly the case in many folic catalyzed cell free systems treatment with Dowex 1 stopped the reaction It was restored by a boiled extract of *E coli*, in turn replaceable by FH_4 A preliminary note confirms these results (Greenberg and Humphreys, 1958)

The conversion of labeled uridine deoxyriboside to thymidine by minced chick embryo or suspensions of bone marrow cells was inhibited by low concentrations of aminopterin, but not the incorporation of labeled thy

midine into DNA CF reversed the aminopterin inhibition and also augmented uridine deoxyriboside utilization in bone marrow cells from ducklings fed a folic deficient diet (Friedkin and Roberts, 1956) Thymidine formation thus represents a third target in higher animals for antifolics (cf Sections II, 6 and IV, 1)

4 *Pantothenic Acid*

Pantothenate or thiamine, combined with methionine, purine, B_{12}, serine, and thymine, helped reverse the toxicity of sulfanilamide for *E coli* (Shive, 1950) More intimations of a connection to C_1 metabolism soon came forth (a) a mutant of *Saccharomyces cerevisiae* required pantothenate, methionine, adenine, and histidine to replace PABA (Pomper, 1952), (b) the interchangeability of PABA and pantothenate for *Bacterium linens* (Purko *et al*, 1954), (c) serine stimulated the production of pantothenate from valine plus β alanine (Altenbern and Ginoza, 1954), and (d) reduced CoA was needed in cell free C_1 transfer systems in which PGA or FH_2 was supplied instead of FH_4 or FH_4 derivatives (Wright, 1958) The site has been located an enzyme from *E coli* catalyzes the reaction VIII (McIntosh *et al*, 1957)



(VIII)

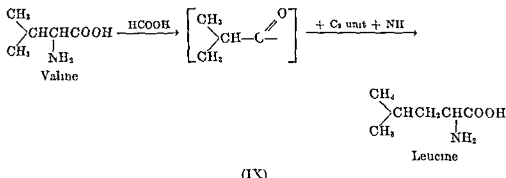
The cofactors have not yet been identified

5 *Valine Leucine*

Participation of C_1 unit in valine leucine biosynthesis again was signaled by inhibition analysis Winkler and de Haan (1948) found that valine along with the usual end products of C_1 metabolism (xanthine, thymine, methionine) overcame sulfanilamide inhibition of *E coli* Valine had a low priority *for FA since its efficacy emerged only in the presence of the afore*mentioned metabolites The differences between these and Shive's (1950) results illustrate how delicate is the detection of metabolites having a low priority for folic coenzymes Several more hints pointed at involvement of C_1 units in valine leucine synthesis (1) an *E coli* that required either methionine or PABA when grown above 37°C was slightly stimulated by leucine and α aminobutyric acid (Bird and Gots, 1955), (2) valine helped spare the PABA requirement of an *E coli* mutant (Davis, 1951) and of a sulfadiazine inhibited wild type strain (Alimchandani and Sreenivasan, 1957) (3) valine, leucine, norleucine, and norvaline were, with methionine, the amino acids

which overcame inhibition of *Mycobacterium tuberculosis* by *p* aminosalicylic acid (Hedgecock, 1958)

Webb (1958) observed that aminopterin inhibited *Aerobacter aerogenes* piled up alanine and valine The interpretation was that both are used for the synthesis of leucine, with the carboxy carbon of valine eliminated as "active" formate (IX)



(IX)

This is compatible with the accepted idea shown above that leucine is formed from the condensation of the isobutyryl moiety of valine with acetate As Webb note , interference by aminopterin with pantothenic acid synthesis would impede CoA synthesis, which would further cripple leucine synthesis

6 Tyrosine Phenylalanine

Kaufman (1958) has briefly described a rat liver preparation which hydroxylated phenylalanine

TPNH + H^+ + O + phenylalanine → TPN^+ + H_2O + tyrosine

FH_4 was highly active in speeding the reaction Anhydroleucovorin had slight activity, PGA, CF, and pteroic acid were completely inactive

As expected, there are microbiological hints that folic acid is concerned in tyrosine metabolism Thus, Lampen *et al* (1949) found that tyrosine, among other amino acids, spared the PABA requirement of an *E coli* mutant The usual folic requiring microorganisms have not provided information on this point because they must be provided with phenylalanine or tyrosine because of other blocks in synthesis Another complication is that the interconversion of these aromatic amino acids may not follow the same path in microorganisms and in higher animals, e g tyrosine and phenylalanine in many bacteria may have a common precursor and hardly be interconvertible

Several clinical phenomena may have their explanations in abnormalities of ring hydroxylation perhaps in the tyrosine phenylalanine reaction or farther along toward melanin synthesis Asenjo *et al* (1958) list hyperpigmentation of the skin, particularly of the face, arms and legs, as one of the

symptoms of tropical sprue, sprue is essentially a folic deficiency (Section XI) Ungley (1955) mentions that in pernicious anemia the urinary excretion of certain phenols is excessive, perhaps this, like the excretion of formiminoglutamic acid (Section III, 3), is a sensitive indication of folic mal

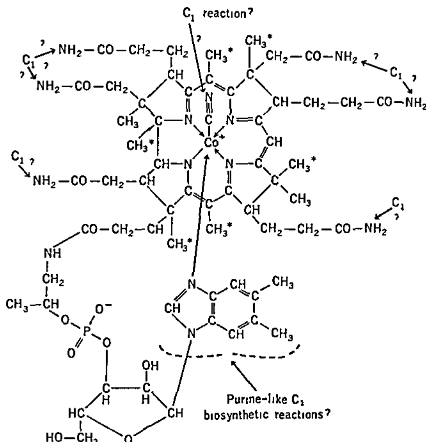


FIG 3 Cyanocobalamin in relation to C_1 transfer in its own biosynthesis Methyls marked with an asterisk are derived from active methyl In the pseudo B_{12} the dimethylbenzimidazole moiety is replaced by purines methyl purines or other heterocycles Factor B is the pigmented moiety lacking the benzimidazole containing nucleotide it is active for *Escherichia coli* 113 3

function An alternate explanation is that folic deficiency impedes the methylation step in adrenaline synthesis

V FOLIC VITAMIN B_{12} INTERRELATIONSHIPS

1 *Generalities*

Major uncertainties about the site of action of vitamin B_{12} promise soon to be dispelled, some folic B_{12} connections should thereby be elucidated As shown in Fig 3, one can postulate a profusion of relations, probable and

less probable, direct and indirect, between FA and B_{12} A variety of nutritional evidence points to a biosynthetic connection between them Shive (1950) independently identified B_1 as a sulfanilamide reversing factor B_{12} similarly spares the PABA requirement of a PABA requiring mutant of *E coli* (Davis, 1952), of a thermophilic bacillus (Baker *et al*, 1955), and of *Acetobacter* strains (Brown and Rainbow, 1956)

Clinical findings underscore a functional connection between FA and B_{12} In pernicious anemia, FA permits a more efficient utilization of B_{12} and sometimes exacerbates the B_1 deficiency through an over abrupt mobilization of B_{12} The reciprocal effect has been reported (Harris, 1956), in two patients, one with Addisonian anemia and the other with nutritional megaloblastic anemia, administration of cyanocobalamin elicited a folic deficiency alleviated by PGA Perhaps the most direct evidence that pernicious anemia patients utilize PGA poorly is the report that formimino glutamic acid is excreted in their urine (Rucknagel *et al*, 1958) These results may have a microbiological counterpart at suboptimal levels of PGA, but not CF, the growth of *Lactobacillus leichmannii* 313 (which requires both FA and B_1) is inhibited by cyanocobalamin (Hausmann and Mulli, 1952, Rogers and Campbell, 1957), confirming results by Greene *et al* (1949)

The finding that supplementing a low B_{12} PGA chick diet with B_{12} or PGA increased the capacity of liver homogenates to convert PGA to CF (Doctor *et al*, 1953) would make it appear that B_{12} was concerned with the reduction of PGA, which leaves to one side the mystery of how B_1 is concerned in the *de novo* synthesis of *N* or *S* methyl groups—the clearest function of B_{12} (Smith 1958)

2 *Methionine Synthesis*

Sparing of B_1 by methionine in higher animals or the complete interchangeability of B_{12} and methionine for certain *E coli* (Kalan and Ceithaml, 1954), points to methionine as having a special relation to B_{12} Methionine metabolism is closely bound to FA as shown by its prominence as a folic sparer as noted earlier in an *E coli* mutant, PABA and methionine are even interchangeable (Bird and Gots, 1958) Convergence of FA and B_1 upon methionine synthesis makes this an attractive system for study

Enzymatic analysis of the *E coli* methionine synthesizing system has progressed *E coli* 121 176, which requires methionine or B_1 , yielded an extract whose synthesis of methionine from homocysteine plus serine was increased six to eightfold by cyanocobalamin (Helleiner *et al*, 1958) The compound formed by mixing equimolar HCHO and FH_4 acted directly as donor of the C_1 unit to homocysteine The system requires (Kisliuk and Woods, 1958a) DPN, ATP, Mg^{++}, hexose diphosphate, and inorganic P

taining δ aminolevulinic acid, 1 amino 2 propanol, threonine, and KCN besides the usual nutrients Only methionine gave rise to labeled B_1. All the activity was in the isoporphyrin moiety—the pigment

b CN The position of CN in metabolism is obscure Its presence in the "pseudo B_1" cofactor described by Barker *et al* (1958) may be an indication that its association with the cobalamins is more than a stabilization artifact Boxer and Rickards (1952) found that cyanide injected into a dog was appreciably converted to thiocyanate There was also an extensive conversion to CO_2 by way of formate, CN carbon also appeared in choline and methionine methyl Perhaps CN metabolism is an important folic problem

c The Benzimidazole Purine Moiety This represents one of the most perplexing and fascinating aspects of B_1 Many cobalamins, inactive for metazoa, are known which, because of various modifications of the benzimidazole moiety, are inactive for metazoa and *Ochromonas malhamensis* but active for various microorganisms (reviews Ford and Hutner 1955, Porter 1957) A bewildering variety of heterocycles can be built into cobalamins by directed synthesis in various microbial systems Among the effective precursors are *o* phenylenediamines (studied especially by Di Marco *et al*, 1957)—which would seem to be proof of the necessary incorporation of a C_1 unit to make the benzimidazole ring Recent work has greatly extended the list of acceptable precursors of the benzimidazole position for various bacteria If unprovided with precursors, most bacteria so far studied make pseudo B_1's, as was learned at great cost in the early industrial production by bacterial fermentation of metazoan active ("true") B_1 Thus *Propionibacterium arabinosum* makes true B_{12} if supplied with 5,6 dimethylbenzimidazole, left to its own devices, it makes an adenine derivative [pseudovitamin B_{12} *sensu strictu* (Perlman and Barrett, 1958)] To the adenine and 2 methyladenine cobalamins originally recognized and identified by several different laboratories as part of "pseudovitamin B_1" and "factor A," respectively, one can add a guanine cobalamin formed by a *Nocardia* (Barchielli *et al*, 1957) and, as a fantastic piling up of ostensible C_1 products, the isolation of a 2 methylmercapto (SCH_3) adenine B_{12} analog from sewage (Friedrich and Bernhauer, 1957) To these conundrums one can add the incorporation into cobalamins by a bacillus and *Staphylococcus aureus* (*Micrococcus pyogenes* var *aureus*) like isolate, of riboflavin, adenine, and 5,6 dimethylbenzimidazole, among other precursors (Southcott and Tarr, 1957)

That methyl adenines, hitherto found only in the bacterially produced pseudo B_{12}'s are fundamental cell constituents, is made likely by the isolation from rat liver microsomes of 6 methylamino and 6 dimethylamino adenine, these, along with 2 methyladenine, were also isolated from *E coli*,

Aerobacter aerogenes, and commercial yeast nucleic acid (Littlefield and Dunn, 1958)

d The Amide Groups The cobalamins have six amide groups, all necessary for biological activity Vohra *et al* (1958) found that HCHO reacts with cyanocobalamin *in vitro*, presumably at the amide groups, to form complexes that could be separated from B_{12} by crystallization, paper chromatography, or paper electrophoresis at several pH's, the complexes were active for *L leichmannii*

An impression from these fragmentary, diverse clues to the connection between FA and B_{12}, is that major pieces in this jigsaw puzzle are missing The cobalamins are already awesomely complex it is humbling only to glimpse a cofactor whose magnitude may be surmised from consideration of ratios such as (1) PABA phosphoribityl formyl polyglutamyl FH_4, (2) pantothenic acid CoA, (3) nicotinic acid TPN, and finally, (4) "true" + "pseudo"B_{12} (+ folic coenzyme?) Co B_{12}

VI Biosynthesis of Folic Acid

1 The p *Aminobenzoic Acid Moiety*

Increased understanding of folic acid function makes the raison d'être of the PABA grouping clearer it imparts resonance stability to the $N^{5,10}$ methenyl bridge compounds This idea is supported by the specificity of PABA The older literature gives the impression that only compounds convertible to PABA are active More recently, 2 amino 5 carboxy pyridine replaced PABA for an *E coli* mutant, the product was active for *Streptococcus faecalis* (Ariens and Simonis, 1955) Wacker *et al* (1958b) found that a group D streptococcus ("*Enterococcus stei*") soon lost sensitivity to *p* aminosalicylic acid, at this point the compound annulled inhibition by sulfanilamide Tracer experiments showed that *p* aminosalicylic acid was incorporated into the cell Extracts of the streptococcus growth with *p* aminosalicylic or salicylic acids were active for *Pediococcus cerevisiae* but not for *S faecalis* *It is remarkable—and a sign of the biochemical times—that* in this instance the PABA sulfonamide inhibition the classical example of a metabolite antagonist relationship, should turn out to be a mixture of simple competition, perhaps lethal synthesis, and directed synthesis Sulfanilamide PGA is chemotherapeutically inactive (Fahrenbach *et al*, 1954) Curiously, PABA has been isolated from an actinomycete produced antibiotic, amicetin, which also contains cytosine and methylserine (Flynn *et al*, 1953) its mode of action is unknown

Knowledge of the biosynthesis of PABA remains about where B D Davis left it in his studies of aromatic biosynthesis A possible intermediate between shikimic acid and PABA may be *p*-aminophenylacetic acid, which

replaced PABA for *Lactobacillus arabinosus* 17 5, shikimic acid was inactive (Nurmikko, 1956)

2 The Pterin Moiety

The biosynthesis of the pterin moiety is very incompletely known Studies of the bacterium *Gaffkya homari* (Aaronson and Rodriguez, 1958) make likely a purine precursor *G homari* has a growth requirement satisfied either by PABA or purines Since the usual supplement of bypassing compounds (thymine, amino acids, etc) was not needed in the absence of PABA, and, unlike *S faecalis*, where folic acid bypassed cells contain no folic acid by *L casei* or *S faecalis* assay (Stokes, 1944a, b), the *Gaffkya* cells grown on purine contained FA active material, it was supposed that purine was acting as precursor, not product A direct proof of the purine precursor theory awaits the demonstration by tracer techniques of the incorporation of several purine atoms into the pterinoid nucleus The flagellate *Strigomonas oncopelti* also uses PABA and purine interchangeably (Newton, 1957), this system has not been analyzed further

Many workers have tried to stimulate folic synthesis by giving a logical seeming precursor pterin such as xanthopterin, together with PABA or *p* aminobenzoylglutamic acid The results have been negative, e g Stokes (1944b), or equivocal The early reports of positive effects of xanthopterin in the treatment of anemias of fish and goats have not been confirmed Korte *et al* (1958b), applying tracer technique, found that cultures of enterococci took up less than 1% of xanthopterin supplied at the 5 mg % level What xanthopterin was taken up was incorporated into a compound oxidizable to 2 amino 4 hydroxypteridine 6 carboxylic acid and so, presumably, into a folic derivative In any event, their tracer and chemical techniques were sensitive enough to measure very minute transfers of carbon atoms from xanthopterin to FA compounds They concluded that the normal synthesis was not through xanthopterin These results seem opposed to those of Katsunuma *et al* (1957) and Katsunuma (1958), who claim that *Mycobacterium avium* forms PGA by condensing *p* aminobenzoylglutamate with xanthopterin and that pteroic acid is inactive (Katsunuma and Shoda, 1957)

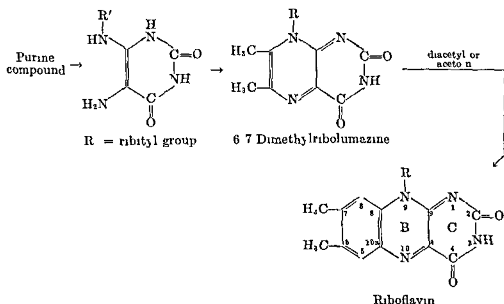
Sevag and Ishii (1958) have set about to identify the fluorescent compounds accumulated in a sulfathiazole inhibited PABA less mutant of *E coli* incubated with PABA Of three fluorescent compounds isolated, one resembled CF, another was assumed to be a reduced pteroic acid derivative, and the third resembled pteroic acid Cell extracts incubated with PABA formed a butanol soluble yellow pigment On hydrolysis, the pigment yielded PABA, glutamic acid, alanine, and leucine, the pigment was inactive for *S faecalis* and the *E coli* mutant A yellow pigment absorbing at

360 mμ was made by washed cells of the mutant incubated with PABA (Ishii and Sevag, 1958) The pigment yielded the same hydrolysis products as the pigment made by the cell free system, it had PABA activity for the mutant and no folic activity for *S faecalis*, *L casei*, or *P cerevisiae* The authors, in reviewing other yellow pigments produced from PABA by microbes, speculate that the pigment is a cofactor for the biosynthesis of FA from precursors rather than being itself converted to folic

The unnamed diphtheroid used by the American Cyanamid team in their isolation of folic acid appears to be absent from type culture collections For biosynthesis studies one of course would be well advised to employ an organism with outstanding ability to make folic acid

3 *Folic Riboflavin Biosynthetic Relations*

Tracer studies and nutritional data on the stimulation of riboflavin synthesis (reviewed by Brown *et al* 1958), show that rings B and C of riboflavin are derived from an intermediate related to the purines by a sequence summarized (XI) by Kuwada *et al* (1958), who isolated ribolumazine intermediates and acetoin from *Eremothecium ashbyii*



(XI)

Probably the same ribolumazines have been isolated by Forrest and McNutt (1958) and Korte *et al* (1958a) Lumazine is 2,4 dioxypteridine (or pterin) Interference with this purine precursor may well explain Schopfer's (1948) finding that sulfanilamide resistant strains of *Eremothecium* tend to be white, not yellow A purine precursor would also account

for the finding that larvae of butterflies injected with labeled glycine and formate yielded labeled leucopterin in the 2, 4, 5, and 7 positions (Weygand and Waldschmidt, 1955) Whether synthesis of unconjugated pterin proceeds via PGA is discussed later (Section VIII)

If pterinoids and riboflavin do compete for a common purine like precursor, then there shoud be a certain reciprocal sparing between pterinoids and riboflavin—complicated by the feedback represented by the folic pterinoids being essential for purine synthesis A systematic study of folic riboflavin B_6 purine relations in the classical strain of *Streptococcus faecalis* ('R", ATCC 8043) has not been made According to Niven and Sherman (1944) this strain does not need riboflavin, nevertheless riboflavin is included in all the basal media recommended for vitamin assays

Nevin (1954) isolated from the mouth streptococci that needed folic acid or riboflavin none of these isolates is listed by the American Type Culture Collection

With FA necessary for purine synthesis, and riboflavin in turn having a purine precursor, Clapper and Meade (1958) reasoned that folic deficiency should induce riboflavin deficiency The riboflavin content of a *S faecalis* strain (derived from a sulfathiazole resistant folic requiring *Streptococcus mitis*) increased as more PGA was supplied, from a low of 3 0 to 50 6 mμg per milligram

The folic requirement of *Crithidia fasciculata* is spared by riboflavin (Nathan *et al*, 1956, Kidder and Dutta, 1958)

It seems reasonable to suppose that there may be many organisms in which riboflavin spares a purine requirement and so, indirectly, a folic requirement The absence of reports of riboflavin mutants in *E coli* perhaps signifies that a riboflavin deficiency in many organisms entails so many other derangements that such mutants are seldom viable

VII Cleavage and Dissimilation of Folic Vitamins

Spontaneous decomposition of folic vitamins, largely a cleavage between C_9 and N_{10}, is greatly accelerated in the FH_4 series, and so has impeded the identification of folic coenzymes This cleavage probably accounts importantly for the fact that only a small part of an administered dose of PGA in man is recoverable from the urine Interest in cleavage reactions has been fostered by two developments (1) the conspicuous cleavage reactions in some microorganisms developing fastness to antifolics, (2) the possibility that such cleavages, spontaneous or enzymatic, figure in the synthesis of physiologically active unconjugated pterins

Liver preparations split added PGA, the split is preceded by reduction to FH_4 compounds (Futterman and Silverman, 1957) Dinning *et al* (1957) estimated the splitting of PGA and CF in man by measuring the appearance

of diazotizable amine in the urine The pterinoids formed spontaneously from FH_2 and FH_4 were identified by Blakley (1957a) as xanthopterin and 2 amino 4 hydroxy 6 methylpteridine

The task of distinguishing between spontaneous and enzymatic cleavages has been undertaken by a group using human red cells Heating (to 100°) and aging of the cells activate an enzyme which degrades PGA or amino pterin, but not amethopterin or CF, at the C_9 N_{10} link, the latter two compounds inhibit the enzyme, xanthopterin was also inhibitory 2 Amino 4 hydroxy 6 formylpterin was produced (Aravindakshan and Braganca, 1958) Further dissection of the system (Braganca and Krishnamurthy, 1958) revealed that the glutathione and Mn^{++}, catalyzing the cleavage, were replaceable by H_2O This makes the reaction a peroxidative one

Clearer evidence for an enzymatic cleavage stemmed from the observa tion that the yeast *Candida tropicalis* grown with high aminopterin and becoming resistant, might show intensely yellow secondary colonies (Nick erson and Webb, 1956) The yellow pigment was butanol soluble It is not clear from Webb (1955) to what extent the yellow pigment consisted of free pterins, he identified the 6 carboxylic acid and the 6 hydroxymethyl derivatives as the main split products of aminopterin, and therefore assumed the primary split product to be the aldehyde which then under went a Cannizzaro reaction

Rauen's (1958) approach to the cleavage problem seems rather com plicated Pig liver homogenates or mitochondria free preparations, in the presence of C_1 donors and oxidizing agents, yielded a mixture of N^{10}-formyl PGA and 2 NH_2 4 OH pteridine 6 aldehyde This apparently combines the enzymatic reduction of PGA to FH_4 and formyl derivatives with their subsequent autoxidation and cleavage, Rauen thinks that the process is nonenzymatic

In a preliminary note, Levenberg (1958) described an extract from an *Alcaligenes* that irreversibly deaminated PGA and other pterins to form the 2,4 dihydroxy (lumazine") derivatives From 2 NH_2-4 hydroxy *pteridine 6 carboxylic acid it formed the corresponding dihydroxy acid* Xanthopterin, aminopterin and leucopterin were inert

High cost and the difficulty of purifying pterins seem to have prevented other uses of the enrichment method for obtaining pterinoid degrading microbes The broadened knowledge of FA function gained by studying the catabolism of histidine and purines promises that studies of the steps in pterinoid degradation by microbes might well contribute ideas if not actual intermediates concerning synthetic pathways The degradations of tryptophan by various bacteria has demonstrated the fertility of this line of investigation

The degradation of PABA follows the usual course for aromatic acids

Durham (1956) isolated p hydroxybenzoic acid as an intermediate from a *Pseudomonas fluorescens* that could utilize PABA as sole substrate The steps from p hydroxybenzoic acid to aliphatic compounds have been traced for pseudomonads in the well known studies of Stanier and his co workers

VIII Pterins

1 Distribution

Distribution of pterins in animals has been reviewed (Fox, 1953, Ziegler Gunder, 1956) A symposium volume (Wolstenholme and Cameron, 1954) has a wealth of information on synthetic methods, structural determinations of completely and incompletely defined pterins, and distribution, especially in insects and fish Figure 4 shows some relationships of pterins to one another and to the folic pterinoids

Pterins are conspicuous in some crustacea, insects, and cold blooded vertebrates These pterins, like the folic pterinoids, are, without exception, 2 NH_2 4 hydroxy substituted Common pterins are xanthopterin (6 OH, orange yellow, yellow green fluorescence), leucopterin (6,7 diOH, white, blue fluorescence), biopterin (6 dihydroxypropyl, buff, light blue fluorescence), isoxanthopterin (7 OH, yellow, violet fluorescence), and erythropterin [6 OH, 7 (COH=COH–CH_2OH)], red The structure of most red pterins is unknown, likewise the complex pterin from human urine, urothione, and the important pterin ("fluorescyanine") from carp scales and silkworm eggs, *Bombyx mori* The literature on pterins records isolations on a heroic scale 6000 liters urine, 100 gm silkworm eggs, 5 kg carp scales, 850,000 butterflies, 500 gm of royal jelly, 5 kg of *Drosophila*, etc The coming of chromatography and microbiological assays to pterin studies was understandably, fervently welcomed

Many investigators have commented on how pterin rich materials are generally accompanied by comparable quantities of guanine or uric acid, and riboflavin, a genetic connection among them has therefore been held highly likely But as uric acid is a waste product, and the grossly visible pterins occur in integumentary structures such as fish and butterfly scales, the thought prevailed that pterins were, like uric acid, metabolically inert end products, and identification of PGA as a pterin was for some years taken to be a biochemical curiosity compatible with the idea of the inertness of the unconjugated pterins

Intimations of an active metabolic role came from a closer examination of insects pterins were also abundant in actively metabolizing tissues Thus the yellow pterin in the female reproductive ducts and testes sheath of certain races of *Drosophila* seemed identical with the yellow pigment in the wild type eyes (Graf *et al*, 1955), the tissues generally of *Drosophila* have a deep violet blue fluorescence (Hadorn and Schwinck, 1956) Identification

as pterins of the water soluble photolabile yellow, and the stable red, eye pigments of *Drosophila* came simultaneously with identification of biopterin as an essential metabolite for a flagellate The whole pterin situation is

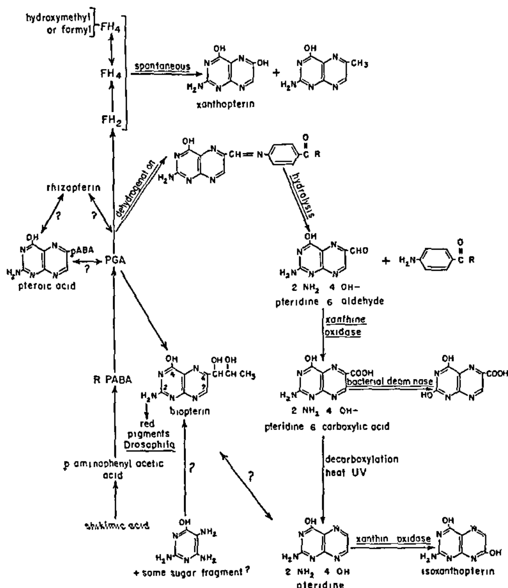


FIG 4 Biosynthetic relations of the folic pterinoids and pterins Several pteridines here and in the text should be designated pterins if the suggestion is followed that the term *pteridine* be restricted to pterinoids not having a fully aromatic nucleus

reminiscent of the carotenoids carotenoids were first known as pigments of fruits and flowers, then some carotenoids were shown to serve as vitamin A precursors and to be indispensable for vision, while vitamin A itself

seemed to be absent from plants But the essential site of action—why is vitamin A deficiency eventually fatal?—remains unknown The analogy between pterin and carotenoids can be extended to vision the eyes of some fish, amphibia, and reptiles contain the pterins xanthopterin and "ichthyopterin" (Pirie and van Heyningen, 1956) This was detected through the brilliant blue fluorescence of aqueous extracts of fish retina and choroid The association of pterins and riboflavin holds here too the pigment epithelium of the retina in some fish contains up to 0 5 mg gm riboflavin wet weight, as compared with the mammalian retina's 3–4 μg gm The photolability of biopterin will be discussed in the next section

Ziegler Gunder (1956) points out that in crustaceans such as the crab, there is a connection of sorts between melanin and pterins—no melanin, no pterin, likewise, in cyprinodont fishes, where there is melanin, there are also pterins There is a close association between "pterinophore" and melanophore cells in fish skin The meaning of its presence in urine—about in 1 part per million (Koschara, 1943)—is unknown except, as discussed later, as a waste product But its effect on the kidney makes this uncertain According to Setsuda *et al* (1956), daily excretion of xanthopterin in urine averaged 4 4 mg in eight normal persons, there was no difference between sexes, but the excretion was increased in pregnancy The xanthopterin output of each patient with chronic myelogenous leukemia, lymphatic leukemia, chronic lymphatic leukemia, and kala azar increased "remarkably" compared with controls

There is no clear genetic relation in *Drosophila* eyes of the water insoluble ommachrome pigments (derived from kynurenine) and the water soluble yellow and red pterins (Hadorn, 1956, Hadorn and Schwinck, 1956)

The distribution of pterins in plants and the origin of isoxanthopterin will be taken up in the next sections

2 *Biosynthesis, Role of Xanthine Oxidase*

As Fig 4 indicates, xanthopterin and isoxanthopterin are probably on divergent pathways Blair (1957), turning from an analysis of the leaf green and the yellow portions of the skin of the green mamba, *Dendroaspis viridis* (besides a yellow nonfluorescent substance, there was the yellow fluorescent riboflavin, isoxanthopterin, and 2 NH_2 4 hydroxypterin 6 carboxylic acid), isolated isoxanthopterin from urine (Blair, 1958a) He did not find the 6 carboxylic acid, and, in a scheme offered for the catabolism of PGA, suggested that isoxanthopterin might originate from biopterin In attempting to trace urinary xanthopterin to its origin, Blair (1958b) found no xanthopterin in normal kidney tissue of the hamster or in transplantable renal tumors induced by stilbestrol Xanthopterin injected into hamsters was converted to leucopterin by xanthine oxidase Leucopterin was not

found in human urine In view of the work of Blakley (1957a) on the spontaneous formation of xanthopterin from FH_2 and FH_4, and of Slavík (see Slavík and Slavíkova, 1958), who found xanthopterin in the urine of rats fed FH_4, Blair concluded that the xanthopterin in urine did not originate in the body tissues

Even if xanthopterin is merely an artifact—a side reaction—one still has to account for its remarkable effect on the kidney As reviewed by Bergel (1956) [this work seems to have been presented earlier only as a series of brief notes e g Haddow (1954)], the kidney of rats and other rodents injected with xanthopterin (150 gm rats received 10 mg intraperitonially) showed a speedy hypertrophy due to a great outburst of mitotic activity in the kidney tubules The compound was excreted into the tubules in the form of leucopterin An identical effect was produced by 4 styryl 2,5,6 triaminopyrimidine Bergel notes here that, in low concentration, xanthopterin is a substrate for xanthine oxidase, at higher, an inhibitor This issue has now sprung into greater prominence Haddow *et al* (1958) report that purified preparations of xanthine oxidase from cow s milk inhibit the growth of certain spontaneous mammary carcinomas in *C3H* and C+ mice The authors wonder whether the effect depends on an increased purine catabolism or the removal of growth promoting pterinoids

Reactions brought about by xanthine oxidase therefore bear directly on the question of the significance of the pterins This enzyme, reviewed by De Renzo (1956), is thought to be complex, partly because it catalyzes two disparate seeming oxidations of simple aldehydes i e , formaldehyde and as its name indicates, of certain purines It is noteworthy that the most powerful inhibitor known of the enzyme is 2 amino 4 hydroxy 6 pteridine aldehyde As is evident from the previous discussion of the cleavage of PGA FH_4 derivatives, both the aldehyde and purine oxidase activities join in these molecules, it is conceivable that, like xanthopterin at very low concentrations, the pteridine aldehyde acts as a substrate for the enzyme

Part of the puzzle of the intimacy between pterins and purines, apart from the likelihood discussed earlier of purines acting as pterin precursors, arises as a result of finding xanthine oxidase to act on both purines and pterins In a detailed study of the specificity of milk xanthine oxidase Bergmann and Kwietny (1958) raised an interesting question Which corresponds to uric acid (the end product of xanthine oxidase activity on purines)—a 2,4,6 or a 2,4,7 trisubstituted pterin? The oxidation of pteridine itself, its monohydroxy derivatives (2,4, and 7) and 2,4 dihydroxypteridine gave in all cases 2 4 7 trihydroxypteridine 6 hydroxypteridine was not attacked Hence they concluded that the 6 hydroxy group of xanthopterin could not originate through the precursors susceptible to xanthine oxidase—which harmonizes with the "spontaneous" origin of xanthopterin from FA

precursors As Bergmann and Kwietny point out, this implies that the leucopterin formed from xanthopterin is a terminal metabolite in the wings of butterflies and plays the same role in pteridine metabolism that uric acid does in purine metabolism

Entirely independent experiments on *Drosphila* in two different laboratories bolster belief in this parallel action Knowing that xanthine oxidase from cream converted 2 NH_2 4 hydroxypterin to isoxanthopterin, Forrest *et al* (1956) identified xanthine oxidase in the pupae of the wild type by the ability of supernates of ground pupae to oxidize this compound to isoxanthopterin, xanthopterin to leucopterin, and xanthine to uric acid The eye color mutants (*maroon* and *maroon like*) lacked the enzyme It becomes apparent why xanthine oxidase should cause parallel changes in pterin eye pigments of *Drosophila*, and their purines The *rosy* mutants of *Drosophila* are deficient in isoxanthopterin (Hadorn, 1956, Hadorn and Schwinck, 1956), they should be deficient in xanthine oxidase As expected, the substrates of the missing enzyme accumulate hypoxanthine and 2 NH 4 hydroxypteridine in *maroon* (Glassman *et al*, 1958), which is also devoid of uric acid, and exactly the same accumulation of hypoxanthine and complete absence of uric acid and isoxanthopterin (Morita, 1958)

The question of the essentiality of xanthopterin can be extended to isoxanthopterin and, for that matter, to xanthine oxidase Hadorn and Schwinck (1956) noted that the growth rate of *Drosophila* stocks homozygous for *rosy* was 53% that of the wild type, with diminished egg production largely responsible This does not tell whether the actual damage is caused by blockage of excretion of uric acid, blockage of isoxanthopterin synthesis, or something else

In other *Drosophila* mutants, deficiency of pterin eye pigments appears to result from lack of substrate, not lack of xanthine oxidase *White apricot* larvae which normally contain very small amounts of 2 NH_2-4 hydroxypteridine and isoxanthopterin, produced isoxanthopterin when fed the hydroxypteridine (Forrest *et al*, 1956)

FA pterins excepted, nothing is known about pterins in bacteria Xanthine oxidase is present in *Lactobacillus casei* (Villela *et al*, 1955) and *Micrococcus lactilyticus* (Whitely and Ordal, 1956)

As may be seen from Fig 4, all the urinary pterins—perhaps all pterins—seem to be derivable, if deviously for some, from FA, especially those originating via biopterin (see next section) Tschesche (1954) remarked that erythropterin, with extra carbons linked at the 7 position, represents a unique biosynthetic puzzle The deviousness of these pathways may explain the failure of Ziegler Gunder *et al* (1956) to obtain more labeling of pteridine (some was observed) when C_2 labeled guanine was injected into *Xenopus* larvae and pterins were then isolated from the skin

Claims have been made that PGA and related metabolites (xanthopterin and a pteridine carboxylic acid) act as a color change hormone in the insect *Carausius* According to Karlson (1956), this work is unconfirmed The studies of Butenandt on royal jelly, discussed in the next section, may however bring the pterins into the ranks of insect hormones

3 *Crithidia Factor and Biopterin*

These pterins occupy a special position biosynthetically, because their side chain at the 6 position means that they cannot be derived from the FA pterins by simple hydrolysis and oxidation reactions and physiologically, because of their relation to photosynthesis, insect vision, and the growth of a protozoan

Crithidia fasciculata, a flagellate parasite of mosquitoes, requires certain unconjugated pterins It seemed to have an extraordinarily high requirement for FA, leucovorin was less effective Liver refined for parenteral use was highly active, but its folic activity, measured by *Streptococcus faecalis*, was negligible (Cowperthwaite *et al*, 1953) The activity of supernatites of cultures of *Ochromonas malhamensis* was retained even after an autoclaving with acid sufficient to destroy folic activity An active fraction was then separated chromatographically from commercial guanosine larger amounts of active material were prepared by recrystallization of guanosine, the mother liquor retained activity From considerations of its probable biogenesis from PGA, stability, and absorption spectrum, *Crithidia* factor was postulated to be an unconjugated pterin with at least one hydroxyl on the side chain (Nathan and Cowperthwaite, 1955) This assumption was soon substantiated a collaborating team at the American Cyanamid Company isolated 20 mg of pale yellow spheres from 4000 liters of human urine (Patterson *et al* 1955) The structure assigned to it (see Fig 4) from the spectroscopic properties of the compound and its degradation products, was proved by synthesis and the configuration of the side chain established, biopterin was 2 NH_2 4 OH 6 [1′ 2′ dihydroxypropyl (L-*erythro*)] pterin (Patterson *et al* 1956) *Attempts to bypass the biopterin requirement* with metabolites known to participate in folic metabolism were negative (Broquist and Albrecht, 1955) 2 NH_2 4 OH 6 substituted pterins were active only if the substituent in the side chain was CH_2OH or a longer aliphatic chain with vicinal hydroxy groups Biopterin was the most active by far 1 0 mμg per milliliter allowed full growth, the hydroxymethyl compound allowed two thirds maximal growth at 0 01–0 03 μg per milliliter The essentiality of PGA or other conjugated pterinoids was evident throughout, but it was needed in far lower concentrations in the presence of the unconjugated pterins Proceeding from the finding that the requirement for *Crithidia* factor became absolute when PGA was bypassed with

thymine (B_{12} was not needed) (Nathan *et al* , 1956) a simplified microbiological assay was devised for biopterin in natural materials Interference from FA was eliminated by preliminary autoclaving of the sample with acid, as was done earlier when assaying *Ochromonas* culture fluids, which destroy conjugated pterinoids but not biopterin (Nathan *et al* , 1958) Kidder and Dutta (1958) have given additional details on the growth requirement of *Crithidia fasciculata*

The issue of the journal that announced the isolation and structure of biopterin also reported the isolation and synthesis of biopterin from the *sepia* mutant of *Drosophila* (Forrest and Mitchell, 1955) Biopterin was accompanied by isoxanthopterin, 2 NH-4 OH pteridine, the 6 carboxylic acid, and N^8 lactyl 7,8 dehydro 2 amino 4 hydroxypteridine The striking photolability of biopterin and the other pterins with hydroxy side chains of course suggested an immediate function in vision This work has been continued by Viscontini and associates in Switzerland, who confirmed the structure of biopterin from *Drosophila,* including trial of its microbiological activity (Viscontini and Raschig, 1958), and undertook to characterize the minor accompanying fluorescent pigments, including "drosopterine" (Viscontini *et al* , 1958, Viscontini, 1958), which is related presumably to the *N* lactyl pterin reported by Forrest and Mitchell It should be noted that these and some other European workers use a different numbering system for the pterinoid nucleus

The scope of biopterin studies widened suddenly Forrest *et al* (1957) reported that the thermophilic blue green alga *Anacystis nidulans* lost activity when an aerated suspension in water stood for a short period at 4° C in darkness The crude leach was light yellow, fluorescent, and yielded 2 NH_2 4 OH 6 carboxypteridine upon oxidation with $KMnO_2$ Pterins were present in relatively high concentration in three other blue greens Van Baalen *et al* (1957) fractionated the extract Two principal pterins were released from acid killed cells, (a) yellow crystalline 2 NH_2 4 OH pteridine (Forrest *et al* , 1957), (b) a blue fluorescent pterin composed of biopterin plus glucose attached to either the 1′ or 2′ of the biopterin side chain (Forrest *et al* , 1958) The pterins amounted, astonishingly, to 0 1% of the dry weight of the algae (Forrest *et al* , 1957) When $C^{14}O_2$ was fed to *A nidulans* during photosynthesis experiments, the C^{14} was incorporated into only the glucose portion of compound C (Van Baalen *et al* , 1957) That the pterins participated in large scale activity such as photosynthesis was suggested by the aforementioned concurrent release of pterins and impairment of photosynthetic ability

This remarkable development impels one to seek parallels in related compounds Katagiri *et al* (1957) isolated a riboflavinyl glucoside from *E coli* and *Aspergillus oryzae* and postulated a fundamental role for ribo

flavin in transglycosidation, Thimann and Radner (1958), investigating the dependence of anthocyanin formation upon light, found that anthocyanin formation in the duckweed *Spirodela oligorrhiza* went on rapidly in the dark in plants supplied with sucrose and riboflavin The main action of light here was supposed to be that of supplying sucrose In view of the uncertainties about light receptors for phototropic and other photic reactions in nonchlorophyllous systems, it seems not unduly speculative to suggest that riboflavin and biopterin might be connected in photoreception as they are elsewhere Royal jelly may make it as proper to discuss pterins under "hormones" as under "vitamins" It makes queen bees out of otherwise sterile workers and fluoresces blue Subsequently, 500 gm of royal jelly fractionated under red light yielded 4 7 mg of chromatographically and electrophoretically pure biopterin (Butenandt and Rembold, 1958) Biopterin was found in all royal jellies and in the larvae of the queen and drones but not in worker's food or in worker larvae Further results one need hardly say, are eagerly awaited

The mode of attachment of the side chain of biopterin suggests a condensation with a carbohydrate, as in its chemical synthesis or with an amino acid, like threonine with the *erythro* configuration The reaction may be analogous to a reversal of the cleavage of L-*erythro* β L *threo* β phenylserine, yielding benzaldehyde and glycine, by an enzyme from the liver and kidney (Bruns and Fiedler, 1958) These authors point out the resemblance to the enzyme that cleaves L-threonine and L-allothreonine to acetaldehyde and glycine The enzyme was considered distinct from serine aldolase in not needing FH_4

Xanthine oxidase does not act on biopterin (Forrest *et al*, 1956) The pattern of urinary excretion of biopterin might be expected, therefore, to differ not only from that of xanthopterin, but also from uric acid The excretion of uric acid is elevated in leukemia perhaps reflecting (Krakoff, 1957) an acceleration of nucleic acid synthesis If this acceleration entailed parallel increases in the folic acid and other cofactors for making nucleic acid, then it would account for the increased excretion of xanthopterin in leukemia noted earlier The turnover of biopterin in leukemia and in normal subjects is one must emphasize, entirely unknown

The fluorescence under ultraviolet of hair infected with the fungi of ringworm has long been used as a diagnostic method An aqueous extract of *Microsporum* infected hair had the absorption and fluorescence features of pterins The extracts were highly active for *Crithidia fasciculata* (Wolf *et al*, 1958) For a brief review of possible pterins in microorganisms including *Corynebacterium diphtheriae*, *Mycobacterium tuberculosis*, and *Aspergilli*, the reader is referred to Wolf (1957) these reports seem too fragmentary to warrant detailed review at this time One might expect to find

xanthopterin and fluorescent lumazines as breakdown products accompanying the growth of organisms prone to make large amounts of riboflavin or FA

IX Evidence for Other Pterinoid mediated Reactions

1 *Steroids*

PGA stimulates the tissue growth response to estrogen in the female chick and rat, correspondingly, aminopterin inhibits the estrogen induced mitosis of the rat uterus, an inhibition reversed by CF (reviewed by Petering, 1952)—one of the rare known functional connections between B vitamins and steroid hormones Doctor (1958) observed an increase in the CF activity in the urine of male rats given estradiol In one experiment, after a dose of 100 μg of PGA, the CF excretion went from the average of 1340 units in the control rats to 2090 in those treated with estradiol, in another group it rose from 7080 to 10,950 units Estradiol inhibited the conversion of PGA to CF in female rats, testosterone inhibited the conversion in castrated male rats and in castrated female rats

Purified preparations of rat liver 3α hydroxysteroid dehydrogenase catalyze the ready interconversion of 3α hydroxy 3 ketosteroids, DPN being the H carrier, the steroids were active at 10^{-6} *M* (Hurlock and Talalay, 1958) Since DPN and TPN are interconvertible, a redox coupling is theoretically possible between pteridines and these steroids

A biosynthetic relation is represented by ergosterol, where the methyl of methionine is the source of its C-24 (Alexander *et al*, 1958, Alexander and Schwenk, 1958) A cell free ergosterol synthesizing system has been prepared from yeast (Parks, 1958) Oxidative removal of angular methyls in the conversion of lanosterol to cholesterol by animal tissues does not appear to be folic mediated (Bloch, 1957)

2 *Miscellaneous*

Nutritional sparing or bypassing provided the initial clues to the functions of FA This technique is not exhausted In the classical *E coli* system (Shive, 1950), the PABA required to overcome sulfanilamide was not eliminated by known metabolites, even though the ratio sulfanilamide PABA reached 30,000,000—which comes dangerously close to the concentration of PABA that can be manipulated without undue disturbance from the PABA which occurs as a contaminant in water and nutrients Other systems may be more informative at this stage The situation in higher animals has been expressed forcefully by Welch (1957) "It is not yet possible in animals to nullify the violently toxic effects of a potent antagonist of folic acid, amethopterin, by supplying a mixture of those compounds known to depend upon folic acid derived coenzymes"

Neither the growth inhibition of the rat induced by dietary sulfaguanidine (Ackerman, 1957) nor the FA deficiency in the mouse induced by excess dietary methionine or glycine (Briggs *et al*, 1958) was reversed by the usual purine pyrimidine amino acid vitamin mixtures 3′5′-Dichloro amethopterin now under trial for the treatment of leukemia seems superior to amethopterin (Goldin *et al*, 1957), it may perhaps yield even more severe deficiencies

Bypassing and antimetabolite experiments with bacteria bring unexpected metabolites into the folic ambit Koft *et al* (1950) found that DL lysine was needed to replace PABA for *Lactobacillus plantarum* (*arabinosus*) 17 5 Reversal of *p* aminosalicylic acid inhibition of *Mycobacterium tuberculosis* required, among other compounds, biotin and C_6–C_{18} fatty acids (Hedgecock, 1958) Redistilled pyruvic and lactic acids spared the PABA requirement of *L plantarum* (Haas and Sevag, 1952) It will be interesting to see whether these metabolites will spare the FA requirement of (a) group F "minute" streptococci where thymidine is ineffective as a bypasser [thymidine is effective with the group G "minute" strains (Felton and Niven, 1955)], (b) *Thermobacterium acidophilum* R 26 where thymidine is ineffective (Løvtrop and Roos, 1957)

The cost of animal experiments makes the possibility of realistic pilot experiments with protozoa an attractive one The use of intact RNA and DNA as folic sparers for the chick (Naber *et al*, 1957) avoids use of the expensive thymidine

In a sense, biopterin was discovered as a folic sparer for *Crithidia fasciculata* There are no published reports on whether it spares the FA requirement of higher animals or whether its urinary output is affected by diet The limited availability of biopterin has precluded large scale experiments aimed at bypassing it However, the intense effort now directed at improving its chemical synthesis promises to make it readily accessible

An attractive experimental object for discerning products of folic metabolism is *Tetrahymena* whose folic requirement has resisted bypassing, thymidine and B_{12} had sparing activity, and folic conjugates were utilized (Dewey and Kidder, 1953) *Tetrahymena*'s great advantage as an experimental object is that it can utilize particulate food such as bacterial bodies This should permit such interesting experiments as feeding it, singly or in combination *S faecalis* and *C fasciculata* growing without FA, to see whether *Tetrahymena* s FA requirement can thus be bypassed

A great variety of folic catalyzed reactions may be brought to light by the transfer of methionine methyl to the methoxyls and some benzene ring methyls of mycophenolic acid produced by *Penicillium brevi compactum* (Birch *et al*, 1958) This is not an isolated instance two angular methyls and the carboxyl of citrinin originate similarly (Schwenk *et al*, 1958)

It is not known whether the degradation of acetone via formate in the

mammal (Sakami and Rudney, 1953) is folic mediated There is no evidence yet from the literature on diabetes that FA is specially involved in the removal of ketone bodies

A C_1 transfer in the synthesis of a carotenoid has been demonstrated for the synthesis of spirilloxanthin by the photosynthetic bacterium *Rhodospirillum rubrum* Of labeled formate added to anaerobic, washed suspensions of the bacterium, 25% appeared in the two methoxyl groups (Braithwaite and Goodwin, 1958), 75% was unaccounted for Spirilloxanthin is the major photosynthetic pigment in *R rubrum* Methoxy carotenoids have not been found outside the photosynthetic bacteria

X Critique of Microbiological Assay Methods

Nearly all microbiological assays for pterinoids have been by means of *Streptococcus faecalis*, *Lactobacillus casei*, *Pediococcus cerevisiae* and, for biopterin, *Crithidia fasciculata* The subject of microbiological assays was reviewed (Hutner *et al*, 1958), therefore this section is brief

The need is acute for a simple, comprehensive microbiological assay for folic compounds One way would be to assay PABA after liberating it with acid or alkali Several potential assay bacteria have been mentioned, e g *Acetobactor suboxydans* and *L plantarum* 17-5 In using *Tetrahymena* for assay purposes one is substituting enzymatic liberation of FA compounds for chemical hydrolysis of FA to PABA, the two methods may be complementary The response of a "thermophilic" *Tetrahymena* (Holz *et al*, 1959) to known bypassing compounds is under investigation The results encourage the hope that a practical procedure is not too remote (Holz, 1958)

As Table I indicates, *P cerevisiae* is clearly superior to *S faecalis* in utilizing 5 formyl FH_4 polyglutamates (Wacker *et al*, 1958a) *L casei* utilizes PGA polyglutamates better than does *S faecalis* That the utilization of polyglutamates may be the crucial attribute in deciding whether an assay organism can be used for the direct detection or measurement of bound and total FA is readily apparent Silverman and Wright (1956) noted that *S faecalis* could not utilize the triglutamyl peptides isolated from *Clostridium cylindrosporum* analogous to CF and N^{10} formyl PGA, they had partial activity for *P cerevisiae* and full activity for *L casei* Much the same problem is encountered with PABA, e g Kandel and Kandel (1955) isolated *p* aminobenzoyl peptides with 10 or 11 glutamic acid residues A multiplicity of folic factors in blood was inferred from the growth responses of the aforementioned lactobacilli (Usdin *et al*, 1956) and isolated from green leaves (Iwai and Nakagawa, 1958), (N^{10} formyl pteric, N^{10} formyl PGA, and N^{10} formyl-PGA glutamic acid) To complicate matters further, Jukes (1955), in reviewing folic assay methods,

noted that certain natural materials inhibit the chick pancreas enzyme used to liberate FA for microbiological assay Further studies on *Clostridium* pointing to folic polyglutamates as the functional coenzymes (Wright *et al*, 1958) emphasize the need for a dependable assay procedure for "total" folic

Familiarity with an assay organism usually uncovers quirks demanding side investigations, this applies even to the classical *S faecalis* When it was transferred a few times on yeast extract acetate glucose, in 6 weeks it responded hardly or not at all to PGA, yet it responded to something in yeast (Jones and Morris, 1949) This observation remains unexplained MasiasR (1956) has noted variations in response to FA depending on whether acetate or citrate pH buffer was used Despite the fact that most of the folic compounds in blood seem to be unavailable to *S faecalis*, e g

TABLE I
UTILIZATION OF 5 FORMYL FH_4 POLYGLUTAMATES BY *Streptococcus faecalis* AND *Pediococcus cerevisiae*

Compound	Growth activity	
	S faecalis	*P cerevisiae* (*L citrovorum*)
5 Formyltetrahydropteroic acid	++	0
5 Formyl FH_4	++	++
5 Formyl FH_4 diglutamic acid	+	+
5 Formyl FH_4 triglutamic acid	±	+++

there was no detectable activity in the serum of humans before the administration of a test dose of PGA (Chanarin *et al*, 1958), the diagnostic test based on *S faecalis* proposed by Girdwood (1956) appears to be dependable for diagnosing folic deficiencies (Chanarin *et al* 1958, Cox *et al*, 1958) The principle of the method is that a significantly low urinary FA activity after an oral dose indicates malabsorption Our experience (unpublished) from comparisons of the *L casei* method with those for formiminoglutamic acid (FGA also called FIGLU ') described earlier (Section III 3) is that the simple *L casei* assay yields results that correlate as well with clinical deficiencies as does the FGA method even though one does not quite know what the bacterium is actually responding to

XI SOME CLINICAL SIDELIGHTS

Three topics of clinical interest are briefly treated here (1) the use of antifolics (2) the chemotherapy of protozoal diseases, and (3) the etiology of sprue

The preceding sections, and some following, emphasize that the behavior of the antifolics impinges on so many phases of folic metabolism that a review of the antifolics would include most of what is known of the mode of action of FA, as is amply demonstrated by a recent symposium volume on the leukemias (Rebuck *et al*, 1957, especially the paper by Nichol, 1957) and several of the reviews cited at the beginning of this review One might attempt to align the advances in knowledge of the pterins with the older knowledge of FA by asking how these advances may contribute to the design of more effective antifolics If the induction of folic cleaving enzymes proves to be important in the development of drug fastness, then the problem arises, how to distinguish quickly between inducer and non inducer compounds? The attention now being devoted to halogenated antifolics arouses curiosity as to whether resistance to cleavage has to do with their high activity Sensitive methods for detection of FA split products in blood or urine would be useful An increase in biopterin excretion might be one such index of cleavage if the limiting factor in biopterin proved to be the cleavage reaction and not the attachment of the side chain Patients receiving amethopterin excrete more CF in the urine (Li *et al*, 1958), perhaps because of a displacement of tissue CF by the drug, presumably there is a concomitant rise in urinary xanthopterin and other products of the spontaneous decomposition of CF and related FH_4 derivatives This complication underscores the potential usefulness of being able to distinguish between spontaneous and enzymatic cleavage products A second mode of inactivation of antifolics is formylation In a system like Rauen's (1958) (Section VII), aminopterin was converted by rat liver homogenates into the nontoxic N^{10} formylaminopterin, with histidine serving as the source of the formyl group, N^{10} formyl PGA was made at the same time (Slavík and Slavíková, 1958) Resistance to the antimalarial pyrimethamine (Daraprim) by *S faecalis*, where the drug interferes with the PGA → CF conversion, exemplifies still another pattern of resistance a strain resistant to 1000 fold the usually lethal concentration bound FA very tightly, unlike the sensitive parent (Wood and Hitchings, 1958)

Pterinoid metabolism is remarkably vulnerable to antimetabolites, especially in protozoa This subject has been reviewed many times, e g by Schnitzer and Grunberg (1957), Goodwin (1958), and Woods and Tucker (1958) The trypanosomid flagellates are notoriously diverse in their response to chemotherapeutic agents Dissection of their growth requirements is revealing a comparable diversity in blocks in pterinoid biosynthesis, the difference between requirements of *Crithidia fasciculata* and the morphologically similar *Strigomonas oncopelti* was noted here earlier The task looming ahead for devotees of these beguiling flagellates is to trace the correlations, if any, between these biosynthetic blocks and their diverse sensitivity to antipterinoids In *C fasciculata* pyrimethamine

competitively antagonizes PGA and CF (Nathan and Cowperthwaite, 1954, Nathan and Funk, 1959) and also competitively antagonizes a *Crithidia* factor active unconjugated pteridine (the trihydroxypropyl derivative—biopterin is dihydroxypropyl) This competitive inhibition of *Crithidia* factor can be demonstrated in the presence of PGA, CF, or thymine (Nathan and Funk, 1959)

Folic malabsorption in sprue is one of its much discussed features Sprue nearly always responds well to massive oral doses of PGA (reviewed by Burch and Ray, 1956, Butterworth *et al*, 1957 Asenjo *et al*, 1958, Gardner, 1958), most cases show a malabsorption of FA Another common symptom is an overgrowth of yeasts, especially of *Candida* sp

One assumption is that the primary biochemical defect is deficiency in the intestinal enzyme liberating FA from its conjugates This enzyme is presumably γ carboxypeptidase, since the glutamyl residues are γ linked This brings up the question of whether the γ glutamyl linkages in FA are unique They are not the capsular glutamyl peptides of *Bacillus anthracis* and *B subtilis* are γ linked (Bovarnick and Bovarnick 1956) As a possible index to the distribution of folic 'conjugase," these bacterial peptides are broken down by aqueous extracts of practically all human tissue, muscle excepted, plasma is inactive The combination of a low folic diet, inadequate intestinal peptidase, and diversion of the enzyme to nonfolic high glutamyl food could account for the initial deficiency

The well known exacerbation of the deficiency by high gluten foods (Anonymous, 1958), would be explicable if the glutamic acid making up as high as 35% of gluten (even higher for certain other cereal proteins) were γ linked We have not found information on this point Incomplete digestion could lead to an invasion of the upper intestinal tract by such organisms as *S faecalis* and *Candida* It is not known whether folic synthesizing strains of *S faecalis* nevertheless absorb FA The splitting of folic conjugates by *Candida* has already been mentioned The abnormal micro flora so induced might intensify the deficiency Burch and Ray (1956), in noting that antibiotics may induce remissions mention the possibility of a competition between exuberant intestinal bacteria and the host The steatorrhea might be of microbial origin

In any event, this and related syndromes (celiac disease, also known as nontropical sprue or idiopathic steatorrhea) call attention to an almost completely neglected aspect of folic metabolism—the glutamyl chains in the native folic coenzymes

XII Prospects and Conclusions

A pressing need is simplification of the *Tetrahymena* and other assay methods for folic acid so that an enzymologist, needing to know whether a likely folic acid cofactor is absent or tightly bound, can buy from reliable

sources the basal assay medium as a dry mix along with a culture from a type culture collection Use of commercially available purified coenzymes, now routine, has greatly speeded the advance of biochemistry A comparable effort seems due for microbiological assays Many pterinoid problems demand a battery of microbiological assays The need is particularly impressive in studying B_{12} folic interactions, e g the clostridial system of Barker *et al* (1958) To illustrate First, is FA concerned? Are there B_6 cofactors? For the pigmented moieties in the "Co B_{12}", there is need of assays for (1) ' true" B_{12} , e g with *Ochromonas malhamensis* and "Lochhead 38", (2) assays comprising also pseudo B_1 's, e g *Euglena, L leichmannii,* and *E coli* 113 3, also perhaps, (3) microbiological assays for purine components

As for the function of biopterin, one should recall that the photolability of riboflavin and PGA were not particularly useful clues Geneticists unraveling the intricate relations of pterin eye pigments of insects would be grateful for an assay for pterins generally adapted to the bioautograph technique, so that one could chromatograph the eyes of a single *Drosophila,* then, by differential microbiological assay combined with fluorescence characteristics, identify all the pterins Is it feasible to prepare a series of pterin requiring bacterial mutants so that differential assays and cross feeding techniques, so successful in elucidating microbial biosynthetic pathways, can be applied? Some microbiological considerations shaping such an enterprise may be mentioned Should one screen first for mutants an abnormally high folic requirement—i e , is it possible to parallel the *Crithidia* investigation with bacteria? Which bacteria to try? Would a physiological anaerobe like *Propionibacterium freudenreichii* best respond to O labile factors? This bacterium probably has a high capacity C_1 transfer system as shown by its use as a B_{12} producer (Pacific Yeast Products, Inc , *1958*), and it is taxonomically close to the diphtheroids, among which high folic producers occur Would such a bacterium respond well to folic conjugates?

Microbial systems that seem suitable may later betray shortcomings *Neurospora* is a good example Harrold and Fling (1952) described mutants of *N crassa* which needed formate or formaldehyde for growth PGA had no effect Since then Swendsied and Nye (1958) cite a personal communication from H K Mitchell that no PGA mutants have been obtained from *Neurospora* An experiment on the reversal of sulfanilamide toxicity for *Neurospora* suggests the reason *Neurospora* cannot utilize exogenous PGA PGA was of limited value in reversing the inhibition but satisfied the PABA requirement of another mutant (Sankar, 1958) One wonders how much of the activity of PGA is attributable to contamination with free PABA For studying PABA synthesis, mutants of *Neurospora* (Zalokar, 1950) which

need sulfanilamide for growth because of an excessive production of PABA, would seem exceptionally suitable

Nutritional dependence on pterinoid antimetabolites is a fascinating phenomenon and part of the problem of resistance to the antifolics in cancer therapy Law (1958) noted that some strains of mouse leukemia have become dependent on antifolics Is the explanation like that for *Neurospora*? If one had antifolic dependent strains of protozoa and bacteria, analysis of their growth requirements might uncover new metabolites, as happened for streptomycin dependent bacteria

Another largely unexploited line of approach is represented by microbes which utilize single carbon compounds as sole source of energy As with the clostridia discussed earlier, one might uncover new group transfer reactions Does the microbial oxidation of *N* methyl or *S* methyl groups require folic coenzymes? As an example one could by selective enrichment obtain microbes which utilize such compounds as trimethylamine as sole source of carbon, nitrogen, and energy If the isolate proved to have an exceptionally high content of FA compounds, as shown by *Tetrahymena* or PABA assay, attempts at a cell free preparation for enzymological dissection would be warranted This sort of analysis has been started for riboflavin by Peel (1958), high riboflavin content of microorganisms went with anaerobic growth, as in clostridia Dac (1958) in studying sarcosine oxidation has demonstrated that such an over all reaction requires careful dissection to find the folic mediated step Another interesting substrate, but rather awkward to use, is methyl mercaptan According to Canellakis and Tarver (1953), methionine forms methyl mercaptan when incubated with liver It was rapidly oxidized to CO_2 and SO_4 after intraperitoneal administration Its carbon also appeared in the β carbon of serine and the methyls of methionine, choline, and creatine, which would indicate an "active formyl" stage The results were considered like those following the feeding of methanol Perhaps the more easily handled dimethylsulfide would yield similar results Quantitatively, the most important C_1 transfer in nature to stretch the definition, is CO reduction in photosynthesis The previously noted presence of biopterin in blue green algae and its attachment to readily labeled glucose would seem to relate pterinoids to photosynthesis Gibbs (1958), in a preliminary communication, to account for the asymmetric distribution of tracer in hexose formed from CO_2, and for the results with *Chlorella* given labeled formate, hypothesized that during photosynthesis CO_2 is reduced to formate and then to HCHO, then transferred to a 5 or 2 carbon acceptor This restates the problem of whether an additional activation of a CO_2 C_1 unit is required to make the fixation of CO_2 by ribulose diphosphate go fast enough One wonders too how this ties in with the excretion of large amounts of glycolic acid by *Chlorella* during

photosynthesis under aerobic condition, and resorption in the dark (Tolbert and Zill, 1956), the relation of folic coenzymes to the glycine glycolic path way in animals was discussed earlier

Limitations of space and knowledge preclude more than a mere listing of interesting virus results involving pterinoids (1) Mice infected with lymphocytic choriomeningitis survive if folic deficient (Haas *et al*, 1957, Levy and Haas, 1958) (2) The activation of bacteriophage into the virulent ("lysogenic") condition can often be induced by irradiating infected bac teria Borek (1956), in seeking a metabolite which, upon irradiation, acted as an inducer, found that of all irradiated products, only leucovorin and anhydroleucovorin acted as inducers Inducing potency resided in the pteridine moiety, since 2 NH_2 4 OH 5 formyl 6 methyl FH_4 yielded inducer upon irradiation, but *p* aminobenzoylglutamic acid did not FH_4 or 10 formyl PGA were also inactive (3) Analysis of psittacosis virus for FA has been attempted (Colón and Moulder, 1958)

A folic B_{12} interaction emerged during an attempt to account for the greatly increased B_{12} requirement in hyperthyroid animals *Ochromonas malhamensis* grown at elevated temperatures likewise showed an enhanced B_{12} requirement, the requirement for extra B_{12} was spared by PGA PGA in turn was spared by purines, pyrimidines, and amino acids (Hutner *et al* 1957) The main nutritional complications caused by elevated temperature simulated those to be expected had sulfanilamide been added to the cultures As *O malhamensis* at ordinary temperatures does not require exogenous PGA and is rich in *Crithidia* factor, temperature stress may also help elucidate the connection between folic and biopterin syntheses Other interesting possibilities are represented by microbes such as *S faecalis* which have a bypassable folic requirement, if they contain biopterin when grown on FA, do they also make biopterin when grown without FA? Particle ingesting protozoa such as *Ochromonas* and *Tetrahymena*, it has been pointed out, may be especially advantageous for dealing with poorly soluble materials

Other complications in pterinoid biosynthetic pathways are hinted at in the unexpected results of Koft and Morrison (1956) on the symbiotic growth of different PABA or PGA requiring bacteria Thus *Acetobacter suboxydans* and *Lactobacillus plantarum*, both requiring PABA, grew well together without PABA Another positive pair was *A suboxydans* and *S faecalis* Ordinarily, if an organism is used as a feeder—supplying an inter mediate—for another, and both are blocked at different points along the same biosynthetic sequence, then the end product has to be supplied to permit some growth, generally this is about 10% of the concentration of end product permitting full growth (Lederberg and Davis, 1950) As noted by Davis, mutual syntrophism (as in the Koft Morrison experiments) is a

special case Is one dealing here with reversible reactions, alternate path ways, or some other complication?

The deepest theoretical value of pterinoid studies may come, as noted, from the glimpses afforded of a remarkable self regulating ("feedback") system for the synthesis of the folic B_{12} catalysts, in which FA and B_{12} participate in their own synthesis, catalyze synthesis of portions in each other, and join in the synthesis of building blocks of ribonucleic acid, deoxyribonucleic acid, and protein How far does the autonomy of this complex extend? *These glimpses have encouraged us to present every* potentially fruitful lead that came to our notice

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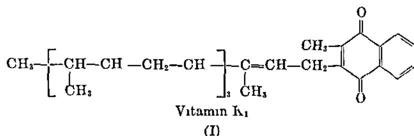
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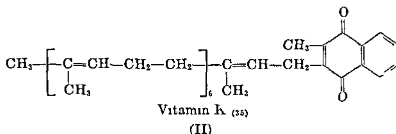
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I Introduction and Nomenclature

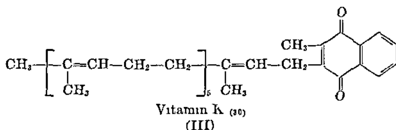
The elucidation of the chemical basis of the K vitamins is due to the schools of Dam, Doisy, and Karrer, who succeeded in 1939 in isolating two pure compounds with vitamin K activity, namely vitamin K_1 (I) from alfalfa and vitamin K (II) from putrefied fish meal The structure of vitamin K_1 was proved by oxidative degradation and by synthesis to be 2 methyl 3 phytyl 1,4 naphthoquinone, the side chain consisting of four



Vitamin K_1
(I)



Vitamin K (35)
(II)



Vitamin K (30)
(III)

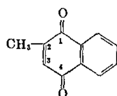
isoprene units ($4 \times 5 = 20$ C atoms) It had been established that vitamin K contains the same ring system with a longer and more unsaturated side chain

Isler *et al* (1958a,b) showed recently by total synthesis that the side chain of *vitamin* K_2 *consists of seven isoprene units* ($7 \times 5 = 35$ C atoms), this is one isoprene unit more than was formerly accepted Thus, Doisy's vitamin K , m p 54°C , has formula II (instead of formula III) and is actually 2 methyl 3 (all *trans* farnesylgeranylgeranyl) 1,4 naphthoquinone

The same group of workers isolated from the mother liquors of vitamin K_2 a small amount of a compound which proved to be 2 methyl 3 farnesyl farnesyl 1,4 naphthoquinone and had thus the structure previously as

signed to Doisy's vitamin K_2 According to a nomenclature proposed by Dam and accepted by Doisy, the two compounds, (II) and (III), will be designated as vitamin $K_{2(35)}$ and vitamin $K_{2(30)}$ The introduction of this second index will facilitate the concise designation of all further homologs to be encountered in nature, such as the vitamin K compounds isolated from mycobacteria (Snow, 1952, Noll, 1958) and from the tissue of mammals fed with C^{14} labeled 2 methyl 1,4 naphthoquinone (Martius, 1958)

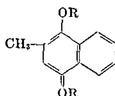
Recent research has shown that 2 methyl 1,4 naphthoquinone (IV) and its derivatives without a side chain in the 3 position, such as (V) and (VI), cannot exert all functions of the K vitamins They either constitute or can be transformed into the necessary exogenous part of the vitamin K mole cule since the animal appears to be able to build on the polyisoprenoid



Menadione

K_3

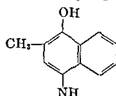
(IV)



Diesters of menadiol

K_4

(V)



2 Methyl-4 amino 1 naphthol

K_5

(VI)

side chain It is evident that the designation of compounds (IV)–(VI) as "vitamins K_3, K_4, and K_5" should be discontinued The generic names "menadione" and "menadiol" will be used in this paper for 2 methyl 1,4 naphthoquinone and 2 methyl 1,4 naphthohydroquinone, respectively

Since the last survey on vitamin K in "Vitamins and Hormones" was written by Dam (1948b), several review articles have appeared, each deal ing with various aspects of the K problem (Dam 1953, Bicknell and Pres cott 1953, Harris *et al* 1954 Jurgens, 1954 Hirschmann, 1955 Martius, 1958) It is the purpose of this paper to summarize the progress in chemistry and biochemistry of the naturally occurring K vitamins and their close ana logs In addition, the paper will review the observed differences in action be tween K vitamins and menadione and an attempt will be made to explain them on biogenetic considerations Menadione like compounds and vitamin K antagonists such as Dicumarol will not be discussed the reader being referred to the comprehensive survey of this subject by Harris *et al* (1954)

II Isolation

1 *Vitamin K_1*

Vitamin K_1 occurs in all green leafy tissues and was first isolated in pure form from alfalfa by Doisy's team and that of Dam and Karrer independ ently (Binkley *et al*, 1939, Dam *et al*, 1939, Karrer and Geiger, 1939,

Karrer *et al*, 1939) At room temperature it is a viscous yellow oil n_D^{20} = 1 5263, $[\alpha]_D^{21} = -0\ 71° \pm 0\ 02°$, dihydrovitamin K_1 diacetate, m p 61–62° Doisy and his collaborators established structure (I) in 1939 by oxidative degradation and by synthesis (see Doisy *et al*, 1941) Color reactions have been reviewed by Dam (1948b), the physicochemical properties are described in Section III of this paper

Recently Schilling and Dam (1958a, b) put forward a stable color reaction with xanthene hydride (5-imino 3 thiono 1,2,4 dithiazolidine) which seems to be especially useful when a large number of samples has to be tested This "Schilling Dam test" is not specific but has definite advantages over the "Dam Karrer test" [reaction with sodium ethylate, (Dam *et al*, 1939, Karrer, 1939)] and the "Irreverre reaction" [with sodium diethyl dithiocarbamate (Irreverre and Sullivan, 1941)] The main problem will always be the separation of the vitamin from interfering coloring matters such as the related benzoquinones

For separation, Schilling and Dam recommend chromatographic filtration through dicalcium phosphate In our laboratories silica gel and silicone impregnated filter paper are the preferred adsorbants for the separation of similar components Decalso and acid alumina are used for crude extracts, petroleum ether and mixtures of petroleum ether (80–105°C) with benzene varying from 10 1 to 1 1 are used for the development and elution of the chromatograms Due to the danger of decomposition on decalso and alumina, these purifications should be performed within 3–4 hours

In preparing the crude extracts dried plant material is carefully ground and extracted with petroleum ether Animal tissues and feces are extracted with acetone without preliminary drying, after removal of acetone, the residue is dissolved in petroleum ether and washed with 95% methanol Up to 50% of the by-products can be removed in this way in the methanol phase (Winterstein and Studer, 1958)

2 Vitamin $K_{2(35)}$

Vitamin K_2 (II), m p 54°C , was isolated from putrefying fish meal by Doisy and his collaborators, employing extraction with petroleum ether, chromatography on decalso, and crystallization at −5°, followed by repeated recrystallization (McKee *et al*, 1939) The same compound was isolated by Tishler and Sampson (1948) from autolyzed cells of *Bacillus brevis* Winterstein adopted Doisy's procedure and modified it by introducing partial sterilization of the fish meal (15 minutes, 115–120°) This results in the survival of a spore forming saprophyte which produces vitamin K_2 in a reasonable yield By this procedure the vitamin was first obtained as yellow crystals melting between 30° and 40° Ten successive recrystallizations raised the melting point to 54° It could be shown by mixed

melting point determinations that this compound was identical with Doisy's vitamin K_2 and different from a synthetic specimen of compound (III) (m p 50°), corresponding to the formerly accepted formula of vitamin K_2 An extensive synthetic effort was required to establish that the side chain of vitamin K_2, m p 54°, consists of seven, and not of six, isoprene units Its identity with a synthetically prepared specimen of 2 methyl 3 (all *trans* farnesylgeranylgeranyl) 1,4 naphthoquinone (II) was finally proved by comparing the melting points absorption spectra, and R_f values as well as the melting points and the absorption spectra of the dihydrodiace tates (Isler *et al*, 1958a b)

It is interesting to note that the production of vitamin K_2 by bacteria varies within wide limits and appears to be very dependent on the compo sition of the bacterial flora Vitamin K_2 can also be produced on artificial media containing aspartic acid, ammonium citrate, and glucose (Winter stein and Studer, 1958)

3 Vitamin $K_{2(30)}$

During the purification of vitamin $K_{2(35)}$, it was noted that the m p 54° could be obtained only by repeated crystallization from different sol vents and that lower melting preparations had a higher extinction than the pure crystals A faster moving component could be detected in these prep arations by paper chromatography (method of Green and Dam, 1954) (see Section III, 6) Separation was finally effected by means of a chromato gram lasting a fortnight, on a column of silicone impregnated cellulose From the first fractions passing through the column on the fourth and fifth days, a new vitamin K_2 compound, m p 50°, was isolated All further frac tions consisted of vitamin $K_{(35)}$ The new vitamin K factor was shown to be identical with compound (III), 2 methyl 3 (all *trans* farnesylfarnesyl) 1,4 naphthoquinone It is thus evident that, besides Doisy's vitamin $K_{2(35)}$, bacteria produce, in smaller amount, the lower isoprenolog with a C_{30} side chain (Isler *et al*, 1958a b)

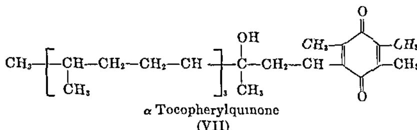
4 Further Vitamin K_2 Compounds

The occurrence of a naphthoquinone derivative in mycobacteria has been described (Francis *et al*, 1949, Snow, 1952, Noll and Jackim, 1958) and seems to be the natural parent compound of Anderson's phthiocol (Ander son and Newman 1933a,b) Noll (1958) succeeded in obtaining it crystalline, with m p 58–59°, starting from *Mycobacterium tuberculosis* (Béremis) The physicochemical data and the spectroscopic studies in ultraviolet as well as infrared regions led to the conclusion that the compound is a higher iso prenolog of vitamin $K_{(35)}$ Its behavior on paper chromatography and its extinction coefficients would be consistent with a C_{50} side chain

Martius (1958) isolated from the tissue of rats fed with C^{14} labeled menadione a labeled K compound which proved to be similar to both vitamin K_1 and K_2. Comparison with synthetic specimens indicated a vitamin K compound with an unsaturated C_{20} side chain. Martius' discovery of vitamin $K_{2(20)}$ in the tissue of mammals fed with menadione is of the utmost importance and will be further discussed in subsequent sections.

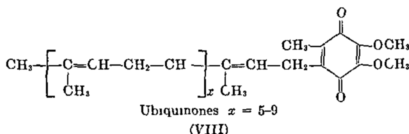
5. Related Benzoquinones

The structural relationship of vitamin K_1 to α-tocopherol and especially to α-tocopherylquinone (VII) was noted already in 1939.



α-Tocopherylquinone
(VII)

The structures of a new class of widely occurring benzoquinones (VIII) containing polyisoprene side chains of different lengths have recently been elucidated and independently named ubiquinones and coenzymes Q by the



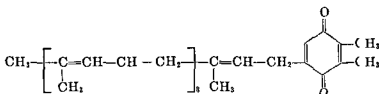
Ubiquinones $x = 5–9$
(VIII)

two groups of investigators (Morton *et al.*, 1957, 1958a,b; Lester *et al.*, 1958; Wolf *et al.*, 1958).

The best known representative, isolated from pig and beef heart muscle, contains a C_{50} side chain and has therefore been designated ubiquinone(50) and coenzyme Q_{10}. A lower isoprenolog, isolated from yeast and with a C_{30} side chain, is called ubiquinone(30). Further compounds isolated from microbial sources contain C_{35}, C_{40}, and C_{45} side chains, respectively. The identity of the side chains of the ubiquinones with those of the vitamin K compounds is remarkable.

Another benzoquinone with a polyisoprene side chain, first described by Kofler in 1946, seems to occur in all green leaves together with vitamin K_1. Kofler's quinone, as well as the ubiquinones, give color reactions similar to those of the K vitamins (positive Dam-Karrer test, Irreverre reaction, and Shilling-Dam test). Its structure has recently been established as an o-di-

methylbenzoquinone derivative (IX) with a side chain of 45 C atoms (Kofler *et al*, 1959a, b, Trenner *et al*, 1959)



Kofler s quinone

(IX)

6 *Conclusions*

The formula of Doisy's vitamin K_2 has been modified to $C_{46}H_{64}O_2$ (II) Contrary to prior assumption its side chain consists of an odd number seven, of isoprene units (7 × 5 = 35 C atoms) It is interesting to note that in this side chain of vitamin K_2 we have the first observed example in nature of a structural part containing a high number of isoprene units all linked together in the normal head to tail sequence Recent research would indicate that this building principle is a very common one

Three isoprenologs of vitamin $K_{2(35)}$ have been isolated, and it is evident that we have to deal with a group of K_2 vitamins differing from each other only in the number of isoprene units making up the side chain

Vitamin K_1 is associated in green plant tissues with Kofler's quinone and in animal tissues with an ubiquinone These very common benzoquinones, devoid of vitamin K activity, give similar color reactions Elimination of these by products prior to any physicochemical evaluation appears to be essential, and all vitamin K contents of natural material reported in the literature may prove to be too high, if not based on bioassay

III Physicochemical Properties

1 *Ultraviolet Absorption*

The quantitative determination of the K vitamins is best achieved by measuring the ultraviolet absorption spectrum which is typical for the naphthoquinone ring system (Fig 1) Vitamin K has the same maxima and minima as vitamin K_1, as have all analogs with the same ring system, but a varying number of isoprene units in the side chain Owing to the higher molecular weight, the extinction coefficients of K_2 are lower than those of K_1 The pure compounds have in petroleum ether a molar extinction coefficient ϵ at 248 mμ of about 1900 The dihydrovitamin K diacetates (Fig 2) have in ethanol a high maximum at 230 mμ, a minimum at 216 mμ and a low maximum at 270–290 mμ, the molar extinction coefficient at 230 mμ is about 87 000 The extinction coefficients ($E_{1\,cm}^{1\%}$) of the pure preparation

proved to be better criteria in the identification of the K vitamins than CH analyses, hydrogenation, and halogenation

2 *Infrared Absorption*

All K vitamins have similar infrared absorption spectra (Fig 3) The bands at 6 0–6 3, 7 7, and 14 0 μ are characteristic for the naphthoquinone ring system, while those at 3 4, 6 9, 7 2, and from 11 to 13 μ are mainly due to the isoprenoid side chains (Saunders and Smith, 1949) It is inter-

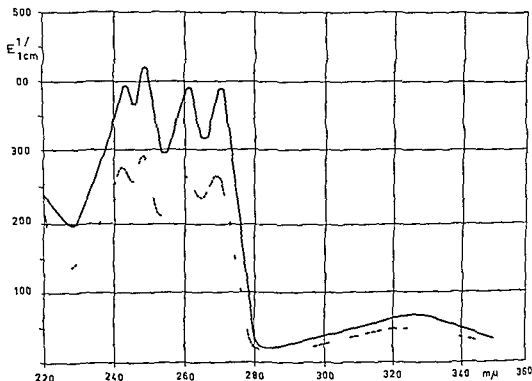


FIG 1 Ultraviolet absorption spectra (in petroleum ether) Vitamin K_1, ——, vitamin $K_{2(35)}$

esting to note that the absorption bands of the side chains are much more pronounced in the crystalline state (spectra 3 and 4) than in the liquid form (spectra 1 and 2) The infrared absorption spectra of the isoprenologs vitamin K $_{(30)}$ and $K_{2(35)}$ can hardly be distinguished from each other

3 *Nuclear Magnetic Resonance*

Nuclear magnetic resonance spectra could have been useful in the structural elucidation and identification of the K vitamins This is demonstrated in Fig 4 by the nuclear magnetic resonance spectrum of vitamin K $_{(35)}$ The different resonances in Fig 4 can be attributed to all the nonequivalent protons in the molecule The resonance at −12 cycles per second (c p s) is

due to the aromatic protons, while that at 55 c p s originates from the olefine protons in the side chain The remaining resonances can be attributed to the different methyl and methylene groups in the molecule The strong resonances at 132 and 143 c p s are due to the methylene and methyl groups in the side chain, whereas the methylene group next to the ring shows up as a doublet at 96 and 103 c p s (von Planta *et al*, 1959)

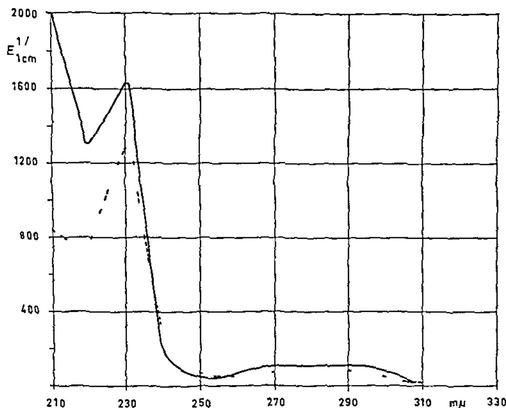


Fig 2 Ultraviolet absorption spectra (in ethanol) Dihydrovitamin K_1 diacetate ——, dihydrovitamin $K_{2(35)}$ diacetate

4 X Ray Diffraction Pattern

This is a very useful method for the identification of the crystalline K_2 compounds, as there are clear differences between isoprenologs In Fig 5 the identical patterns of natural and synthetic vitamin $K_{2(35)}$ as well as the different pattern of vitamin $K_{2(30)}$ can be seen (Isler *et al*, 1958b) These patterns show that the unit cell of the $K_{(30)}$ lattice has a lower symmetry than that of the $K_{(35)}$ lattice

5 Optical Rotation

Vitamin K_1 isolated from natural sources has a very low optical rotation which can be interpreted theoretically (Beredjick and Schurch, 1957)

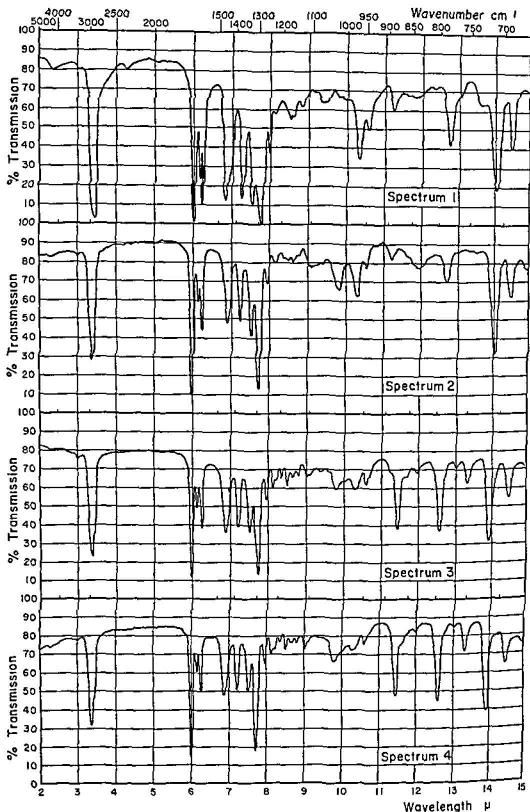


FIG 3 Infrared absorption spectra of the K vitamins Spectrum 1 vitamin K_1 (oil) spectrum 2 vitamin $K_{2(30)}$ (molten) spectrum 3 vitamin $K_{(30)}$ (crystalline) spectrum 4 vitamin $K_{2(35)}$ (crystalline)

FIG 4 Nuclear magnetic resonance spectrum taken in CCl_4 solution with benzene as internal standard at a radiofrequency of 25 megacycles The chemical shifts are given in cycles per second

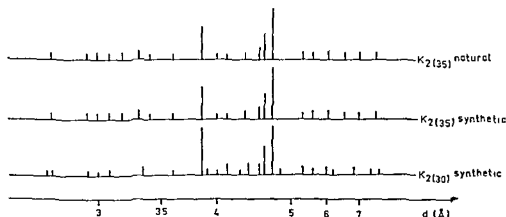


FIG 5 X Ray diffraction patterns of vitamins $K_{2(30)}$ and $K_{2(35)}$ The lattice spacings and intensities were measured with a Geiger counter goniometer and copper K_α radiation

phytylating agent More recently Isler and Doebel (1954) have shown that boron trifluoride etherate is a more efficient catalyst In addition they found that ethers and esters of phytol, as well as isophytol and its derivatives, can be employed instead of phytol

The most efficient syntheses reported so far start from menadiol 1 monoesters (Fig 8) These are allowed to react with phytol in the presence of boron trifluoride etherate, aluminum chloride, or potassium acid sulfate as catalyst (Hirschmann *et al*, 1954, Kvita *et al*, 1957, Lindlar, 1958) The unchanged menadiol 1 monoester can be removed by precipitation with

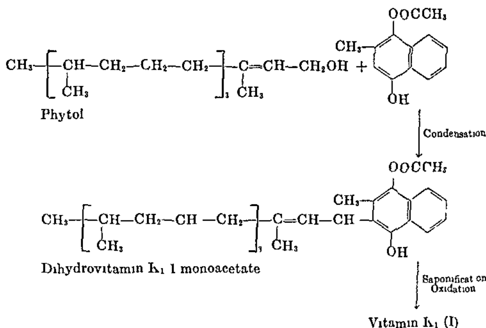
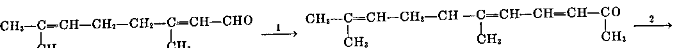


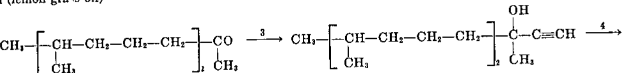
FIG 8 Vitamin K_1 from phytol and menadiol 1 monoacetate

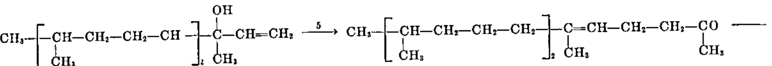
petroleum ether and filtration The condensation product, dihydrovitamin K_1 1 monoacetate, which is soluble in petroleum ether, can be extracted into Claisen's alkali This operation effects hydrolysis of the ester group and is followed by oxidation to the quinone The over all yield is reported to be as high as 66 %

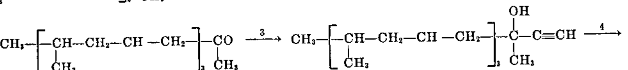
2 Racemic Vitamin K_1 from Isophytol

Racemic isophytol was synthesized from lemon grass oil, or totally from acetone In the synthesis of isophytol from lemon grass oil (Fig 9) its main constituent, citral, is condensed with acetone to give pseudoionone Hydrogenation leads to hexahydropseudoionone Lengthening of the chain by an isoprene unit is effected by condensation of this ketone with sodium acetylide in liquid ammonia followed by partial hydrogenation of the triple









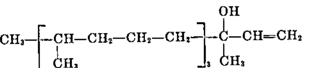


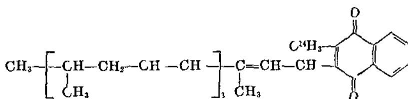
FIG 9 Synthesis of isophytol from citral Numbers along arrows indicate *1* condensation with acetone *2* hydrogenation *3*, sodium acetylide in liquid ammonia *4* partial hydrogenation *5* phosphorus tribromide and acetoacetate synthesis or condensation with diketene followed by pyrolysis

tically active isophytol by ozonolysis to octahydrofarnesylacetone which was followed by condensation with acetylene and partial hydrogenation. Its condensation with menadiol followed by oxidation and reductive acetylation led to dihydrovitamin K_1 diacetate, m.p. 61–62°, which appeared to be identical in every respect with natural vitamin K_1. It was concluded that the slight differences observed between vitamin K_1 from phytol and rac. vitamin K_1 from synthetic isophytol (rotation, crystallizing properties of the dihydrodiacetates) are due to the asymmetric C atoms in the side chain. A further conclusion was that the double bond of both products and of all analogs seems to have the same configuration, most probably the *trans* configuration.

3. C^{14}-Labeled Vitamin K_1

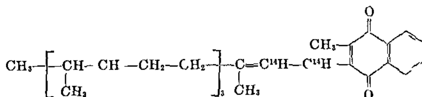
Labeled vitamin K_1 is an important tool for the elucidation of its function and its metabolic fate. C^{14} can be introduced either into the naphthoquinone ring system, into the methyl group in the 2 position, or into the phytyl chain in the 3 position. For the synthesis of labeled menadione, reference should be made to the handbook of Murray and Williams (1958).

Lee *et al.* (1953) synthesized rac. vitamin K_1 (X) by condensing menadiol labeled in the methyl group with isophytol according to the procedure of Isler and Doebel (1954). They obtained rac. vitamin K_1 with a specific activity of 0.08 microcurie per milligram.



2-(Methyl-C^{14})-3-phytyl-1,4-naphthoquinone

(X)



2-Methyl-3-(phytyl-1',2'-C^{14})-1,4-naphthoquinone

(XI)

Wursch (1958) prepared C^{14}-labeled isophytol by condensing octahydrofarnesylacetone with C^{14}-labeled acetylene; this was followed by partial hydrogenation of the triple bond. This isophytol was condensed with menadiol-1-monobenzoate according to Lindlar's procedure (Fig. 11) to give dihydrovitamin K_1 monobenzoate which was transformed into labeled

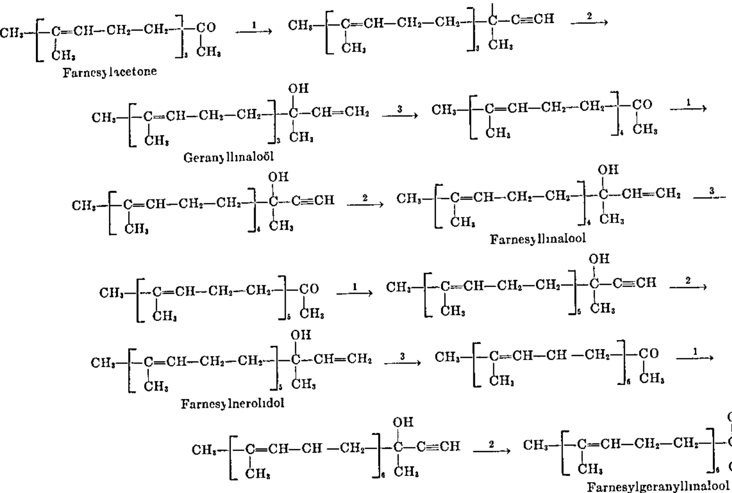


Fig 12 Synthesis of farnesylgeranyllinalool from farnesylacetone *1* Sodium acetylide in liquid ammonia *2* partial hydrogenation *3* phosphorus tribromide followed by acetoacetate synthesis

rac vitamin K_1 (XI) with a specific activity of 0 7 microcurie per milligram Very much higher activities could easily be obtained by the same method of preparation It has to be noted, however, that radioactive vitamin K_1 decomposes relatively rapidly under the influence of its own radiation (Woods and Taylor, 1957)

4 Vitamin $K_{2(35)}$ from all trans Farnesylgeranyllinalool

The synthesis of the side chain started from acetone and followed the pattern of the isophytol synthesis in Fig 9 *Cis trans* isomerism at the newly formed double bonds must be taken into account Geranylacetone was sep

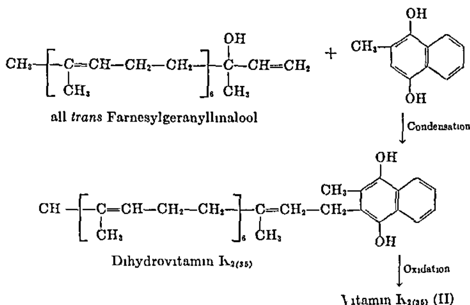


FIG 13 Vitamin $K_{2(35)}$ from all *trans* farnesylgeranyllinalool and menadiol

arated into the pure *cis* and *trans* form by careful fractional distillation Lengthening of the pure *trans* form was achieved via farnesyl bromide by acetoacetic ester synthesis In this way, the newly formed double bond has about 90% *trans* and 10% *cis* configuration, whereas on pyrolysis of the diketene adduct about two thirds *trans* and one third *cis* forms are obtained

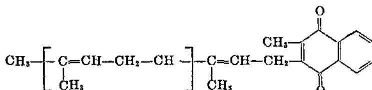
The farnesylacetone formed was separated into the mono *cis* and the all *trans* form by chromatography of its semicarbazones on alumina The synthesis of all *trans* farnesylgeranyllinalool from all *trans* farnesylacetone (Fig 12) consists in lengthening the side chain three times by an isoprene unit and isolating each time the pure all *trans* form of the C_{23}, C_{28}, and C_{33} ketones by chromatography of their semicarbazones on alumina The all *trans* form of the C_{33} ketone was finally lengthened to all *trans* farnesyl geranyllinalool, which was condensed with menadiol (Fig 13)

The purified product crystallized immediately on seeding with natural

vitamin K_2 Its identity with Doisy's vitamin K_2 isolated from natural sources was shown by comparing the melting point (54°), absorption spectra, and R_f values, as well as the melting points and absorption spectra of the dihydrodiacetates The mixed melting points showed no depression (Isler *et al*, 1958a,b)

5 *Vitamin K_2 Series*

The intermediates in the synthesis of all *trans* farnesylgeranyllinalool listed on the right in Figs 10 and 12, namely methylbutenol (C_5), linalool (C_{10}) nerolidol (C_{15}), geranyllinalool (C_{20}), farnesyllinalool (C_{25}), and farnesylnerolidol (C_{30}) were condensed by Isler *et al* (1958a,b) with mena-



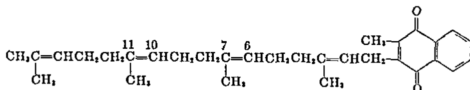
Lower isoprenologs of vitamin $K_{2(35)}$ $x = 0–5$
(XII)

diol and transformed into the lower vitamin K_2 isoprenologs of the general formula (XII) by following exactly the procedure outlined in Fig 13

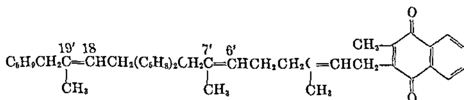
All compounds were purified by chromatography and, when possible, by repeated crystallization Their purity was checked by measuring the molar extinction coefficient at 248 mμ and that of their dihydrodiacetates at 230 mμ The R_f values of these compounds, which decrease on lengthening the side chain, are listed in Fig 6 together with the melting points

2 Methyl 3 (all *trans* farnesylfarnesyl) 1,4 naphthoquinone (III), m p 50°, gave a melting point depression with Doisy's vitamin $K_{2(35)}$ and proved to be identical with the faster moving component of its mother liquors isolated by Winterstein from putrefied fish meal (see Section II 3)

Isler *et al* (1958a,b) synthesized, in addition to the all *trans* compounds four mono *cis* forms (XIII–XVI) with a C_{20} and a C_{30} side chain respectively the *cis* bond being either in the 6′,7′ position (nearer to the naphthoquinone ring system) or in the 10′,11′ and 18′,19′ position respectively (near to the end of the side chain) In contrast to the corresponding all



2 Methyl 3 (6 7 mono *cis* geranylgeranyl) 1 4 naphthoquinone (XIII)
2 Methyl 3 (10 11 mono *cis* geranylgeranyl) 1 4 naphthoquinone (XIV)

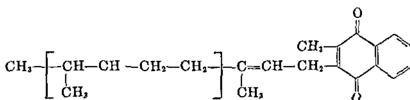


2 Methyl 3 (6′ 7 mono *cis* farnesylfarnesyl) 1,4 naphthoquinone (XV)
2 Methyl 3 (18 ,19 mono *cis* farnesylfarnesyl) 1 4 naphthoquinone (XVI)

trans forms, these mono *cis* compounds could not be obtained in a crystal line state It is noteworthy that the R_f values and the absorption spectra *of the stereoforms proved to be nearly identical with those of the all trans* form, whereas the biological activities varied considerably, as will be shown in Section V 5

6 Related Compounds

The vitamin K_1 series (general formula XVII) were synthesized by Isler *et al* (1953) for comparison of their properties with those of vitamin K_1 as well as with the corresponding compounds (XII) of the vitamin K_2

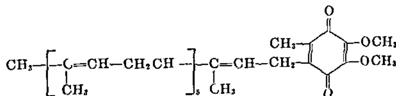


Isoprenologs of vitamin K_1 $x = 0$–5
(XVII)

series The same authors synthesized some analogs whose side chains were aliphatic, alicyclic, or contained a benzene ring Their action as antagonists of Dicumarol proved to be less reliable than that of the compounds of the vitamin K_1 and K series

For a complete list of further analogs and of the menadione like compounds, reference is made to Doisy *et al* (1941) and Deuel (1951)

The recent synthesis of ubiquinone(30) (XVIII) from yeast by Gloor *et*



Ubiquinone(30)
(XVIII)

al (1958) is noted, as it contains the same side chain as vitamin $K_{2(30)}$ The

simpler analog of (XVIII) with a phytyl chain was synthesized by Morton *et al* (1958a) and by Shunk *et al* (1958)

7 Conclusions

Vitamin K_1 is best synthesized by condensation of a menadiol 1 ester with phytol or isophytol and subsequent saponification and oxidation Vitamin K_1 synthesized from phytol appears to be identical with the natural product, vitamin K_1 from synthetic isophytol differs slightly in having a racemic phytyl chain The double bonds in β,γ position of all vitamin K compounds have the same, most probably the *trans* configuration

The structure of vitamins $K_{2(35)}$ and $K_{(30)}$ are established by total synthesis The finding that both are all *trans* compounds is quite in agreement with the all *trans* configuration of natural squalene

The introduction of one double bond with *cis* configuration can alter in the vitamin K_2 series some physicochemical properties as well as the biological activity markedly, as shown by comparison of some mono *cis* forms with the corresponding all *trans* compounds

V Biological Evaluation

1 Methods

The classical method for the biological testing of vitamin K active substances is the measuring of their effect on the blood clotting time of the vitamin K deficient chick Dam *et al* (1951) have described a modified method, by which a linear relationship between vitamin K dosage and clotting time was obtained

In a second method the antidotal effect of vitamin K to Dicumarol and related substances in different laboratory animals such as rats rabbits dogs has been employed and the first quantitative results have recently been published by Lowenthal and Taylor (1958), with the rat as the test animal

Concerning the mode of action of anticoagulants like Dicumarol good evidence is given in a study by Dam (1948a), that the same disturbance in the blood clotting mechanism is occurring here as in vitamin K deficient animals A mixture of the plasmas from vitamin K deficient chicks and from Dicumarol poisoned chicks possessed about the average blood clotting time of its components If the factors, lacking in the two kinds of plasma were different the mixed plasma should clot almost as quickly as normal chicken plasma There is some evidence, however, that vitamin K deficient plasma is also lacking in a second factor, and Dicumarol poisoned plasma in a third (Dam and Søndergaard 1948) These findings were confirmed by Quick *et al* (1952) using dogs instead of chicks

In man no difference in the disturbance of the clotting mechanism

brought about by vitamin K deficiency and by administration of Dicumarol, could be detected (Naeye, 1950) In both cases the plasma thromboplastin component (PTC) was reduced and could be returned to the normal level by administration of vitamin K_1

2 Activity of Vitamin K_1 and Menadione in Vitamin K Deficiency

Dam and Søndergaard (1953a) found that in the usual test with vitamin K-deficient chicks, using an assay period of 20 hours or more, the activities of vitamin K_1 and menadione are approximately equal on a molar basis However, on intravenous administration and measurement of the prothrombin time within the first 2 hours, it could be shown that vitamin K_1 acts more rapidly than menadione There was no difference between vitamin K_1 and menadione after 2 hours

Equal activities for vitamin K_1 and menadione were found by Fisher *et al* (1956) in the dog, where vitamin K deficiency had been produced by chronic lack of bile On oral administration the water soluble menadione was more active than the oily solution of vitamin K_1

Similar differences in activity between menadione bisulfite, menadione, and vitamin K_1 have been observed in chicks under practical feeding conditions, and most probably are due to better absorption of the water soluble forms (Frost and Spruth, 1955, Frost *et al*, 1956, Perdue *et al*, 1957a,b)

3 Activity of Vitamin K_1 and Menadiones as Antidotes against Dicumarol and Related Substances

Contrary to the full activity of menadiones in vitamin K deficient animals, they are much less active than vitamin K_1 in normalizing the prolonged blood clotting times caused by Dicumarol and other anticoagulants This was first shown in rats by Smith *et al* (1946) and Smith (1947) In numerous investigations on animals (Collentine and Quick, 1951, Dam and Søndergaard, 1953b) and on humans (Ley *et al*, 1951, Van Itallie *et al*, 1951 Douglas and Brown, 1952, Toohey, 1952, Rotter and Meyer, 1952, Larrieu Boubée, 1952, Sturup, 1955) the superiority of vitamin K_1 over menadione in counteracting the anticoagulant effect of Dicumarol and related compounds has been confirmed

In an extensive study, Isler *et al* (1953) examined a great number of vitamin K_1 and K_2 derivatives for their antidotal action to different anticoagulants It was clearly established that a side chain of a minimum length is necessary for this activity

Lee *et al* (1950) administrated C^{14} labeled Dicumarol to rats They found that it was stored in the liver and that the prolongation of the prothrombin time was parallel to the stored Dicumarol The decrease of Dicumarol in the liver was accelerated by menadiol diphosphate From this

it is concluded that menadiones, too, have some antidotal action to Dicumarol

4 *Activity of Vitamin K_1 and K Analogs*

A number of isoprenologs in the vitamin K_1 and K_2 series, the syntheses of which are described in Section IV, 5 and 6 were tested in the vitamin K deficient chick Biological activity in either the vitamin K_1 or the vita

TABLE I

BIOLOGICAL ACTIVITY OF THE VITAMIN K_1 SERIES

Side chain in 3 position		Number of chicks	Activity (vit K_1 = 100%) (%)
C atoms	Designation		
5	Dimethylallyl	13	<5
10	Dihydrogeranyl	10	Approx 10
15	Tetrahydrofarnesyl	12	Approx 30
20	Phytyl	11	100
25	Octahydrofarnesylgeranyl	12	Approx 80
30	Decahydrofarnesylfarnesyl	9	Approx 50

TABLE II

BIOLOGICAL ACTIVITY OF THE VITAMIN K_2 SERIES

Side chain in 3 position		Number of chicks	Activity (vit K_1 = 100%) (%)
C atoms	Designation		
10	Geranyl	9	Approx 15
15	Farnesyl	8	Approx 40
20	Geranylgeranyl	15	100
25	Farnesylgeranyl	11	Approx 120
30	Farnesylfarnesyl	15	100
35	Farnesylgeranylgeranyl	12	Approx 70

min K series was found to be clearly dependent on the length of the side chain (Wiss *et al*, 1959) In the vitamin K_1 series, it was the natural form of vitamin K_1 with a side chain of 20 C atoms which showed the maximum effect (Table I)

In the vitamin K_2 series the maximum effect was found in the vitamin K analog with 25 C atoms in the side chain (Table II) The activity of this vitamin K_2 form was even somewhat higher than that of vitamin K_1 (Wiss *et al* 1959) This may serve as a hint that in addition to vitamin $K_{2(35)}$, $K_{2(30)}$, $K_{(\ 0)}$ (Section II, 3 and 4) this form *may also occur in nature*

It has been found that a cytochrome c reductase preparation, dea by extraction with isooctane, can be reactivated by addition of vit and derivatives (Nason and Lehman, 1956) As described in Secti 2, other substances with an isoprene like side chain, such as ubic vitamin K_1, and K were found to be active Additionally it could b that this side chain—and not the redox system—is responsible for activation (Weber *et al*, *1958a*,b)

On comparing the biological activities of the above mentioned K_1 and K_2 isoprenologs with their reactivation effects on extracte chrome c, a good correlation was found Especially in the vitamin K there is a very close conformity between these two activities (Wei Wiss, 1959) (Fig 14)

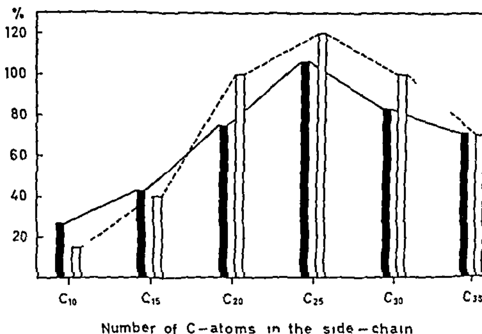


FIG 14 Activity of the vitamin K_2 series White columns biological act the chick Black columns reactivation of the cytochrome c reductase

5 Stereochemical Influences on the Activity of Vitamin K_1 and K

Vitamin K_1 obtained by total synthesis has a racemic phytyl side and differs in this respect from the vitamin K_1 isolated from natur terial It could be shown, however, that there is no difference in the b cal activity in the vitamin K-deficient chick between the two forms *et al*, 1959) (Table III)

In the vitamin K_2 forms and their isoprenologs geometric isome possible owing to the double bonds present in the side chain The all the 6′,7′ mono *cis*, and the 18′,19′ mono-*cis* forms of vitamin $K_{2(30}$

tested on vitamin K deficient chicks No difference was found between the all *trans* and the 18′,19′ mono *cis* compound The 6′,7′ mono *cis* isomer, however in which the double bond is near the ring, was significantly less active than were the other two (Table IV)

TABLE III

BIOLOGICAL ACTIVITY OF VITAMIN K_1 WITH A RACEMIC SIDE CHAIN AND VITAMIN K_1 WITH OPTICALLY ACTIVE SIDE CHAIN (FROM NATURAL PHYTOL)

Chicks	Compound	Number of chicks	Prothrombin time (in seconds)
Normal	—	4	50 ± 2 2[b]
Vit K deficient	—	26	>180
Vit K deficient	Synthetic vit K_1	13	87 ± 7 1
Vit K deficient	Natural vit K_1	13	85 ± 7 4

Dosage 56μg per kilogram of body weight
[b] Mean ± standard error of the mean

TABLE IV

BIOLOGICAL ACTIVITY OF THE GEOMETRIC ISOMERS OF VITAMIN K_2 WITH A C_{20} AND C_{30} SIDE CHAIN

Side chain in 3 position		Number of chicks	Prothrombin time (in seconds)	Significance
C atoms	Configuration			
	all *trans*	14	86 ± 5 7	
20	6 7 mono *cis*	14	118 ± 10 3	$p = 0\ 02$
	10 11 mono *cis*	11	85 ± 7 7	
	all *trans*	13	58 ± 4 4	
30	6 7 mono *cis*	19	133 ± 6 8	$p = 0\ 001$
	18 19 mono *cis*	19	52 ± 1 6	

Mean ± standard error of the mean

From the isomers of the vitamin K_2 analogs with 20 C atoms in the side chain the activity of the 6′,7′ isomer was also clearly reduced compared with the all *trans* and 10′,11′ mono *cis* compound (Wiss *et al*, 1959) (Table IV)

As mentioned in Section V, 4 a good correlation between the biological activity and the reactivation of cytochrome c reductase of the different K_1 and K_2 analogs was found The same could be said for the above described isomers of vitamin $K_{(30)}$ (Fig 15) and the K_2 analogs with 20 C atoms in the side chain (Weber and Wiss, 1959) (Fig 16)

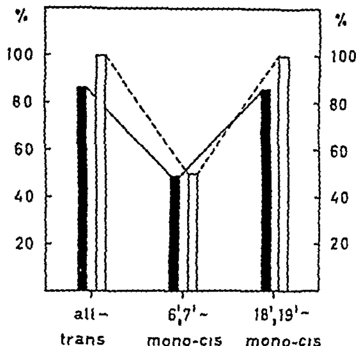


FIG 15 Activity of the geometric isomers of vitamin $K_{2(10)}$ White columns biological activity in the chick Black columns reactivation of the cytochrome c reductase

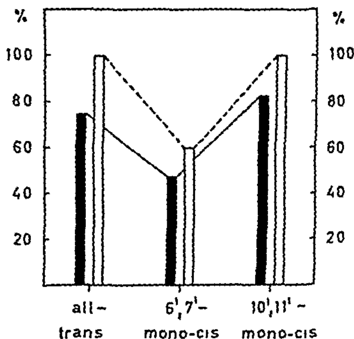


FIG 16 Activity of the geometric isomers of vitamin $K_{2(20)}$ White columns biological activity in the chick Black columns reactivation of the cytochrome c reductase

6 *Conclusions*

There is little doubt that the symptoms produced by vitamin K deficiency and by poisoning with Dicumarol and related substances are very similar In both cases, administered vitamin K_1 acts by normalizing the prolonged blood clotting time Menadiones, however, are only as active as vitamin K_1 in nutritional vitamin K_1 deficiency and are almost without effect as antidotes to Dicumarol

As mentioned in Section VI, Martius (1958) provided evidence that menadione is transformed in the animal body into a vitamin K_2 derivative by the addition of an isoprene like side chain of 20 C atoms From observations described in Section VII, 2, it would seem that vitamin K_1, unlike menadione, possesses enzymatic activity in the respiratory chain phosphorylation

Assuming therefore that only vitamin K substances with a typical side chain, as in vitamin K_1 and the forms of vitamin K_2, are active in metabolism, the above mentioned differences in the biological activity of vitamin K_1 and of menadione can be explained Menadiones, necessary only in very small doses to cure nutritional vitamin K deficiency, may be quantitatively transformed into the active vitamin K_2 form This biosynthesis being limited, however, as described in Section VI, is insufficient to provide the high amount of metabolically active vitamin K_2 needed to counteract Dicumarol poisoning

It could be shown that there is a clear dependence of biological activity on the length of the side chain and that there exists a very close relationship between the *in vivo* activity in the vitamin K deficient chick and the *in vitro* effect on cytochrome c reductase This supports the view that binding of the enzymatically active form on the mitochondria is determined by the side chain and that this function becomes the limiting factor for the *in vivo* activity, too

As mentioned above, it is probable that menadione acts in preventing vitamin K deficiency only by its conversion into a vitamin K form containing an isoprene like side chain Together with the fact that menadione itself could not be found in nature, it seems logical to accept a vitamin K_1 or a vitamin K form as the biological standard In view of its easy availability, vitamin K_1 would seem to be the most appropriate

VI Biosynthesis

The discussion of this topic will be limited to the special problem of the synthesis of a vitamin K_2 derivative from menadione in the animal body, since nothing is known about the synthesis of the ring component of vitamin K_1 or K_2 by the plant or the bacterial cell

After administration of C^{14} labeled menadiol diacetate to vitamin K

deficient chicks over a long period, Martius (1956) found that the labeled C is incorporated into a substance which, after distribution between two organic solvents, shows a behavior quite similar to that of vitamin K_1. Later this substance was identified as a vitamin K_2 compound with a side chain of 20 C atoms (Martius and Esser, 1958). From this experiment no information about the mechanism of the side chain synthesis is available. Investigations on the biosynthesis of ubiquinones may give some information on this point, as the side chain of this substance has the same structural arrangement. It was found that labeled mevalonic acid is incorporated into the ubiquinone of rat liver (Gloor and Wiss, 1958). As there is little doubt that the ring component (2,3-dimethoxy-5-methylbenzoquinone) is essential for the animal, mevalonic acid seems to be used only for the synthesis of the isoprene-like side chain.

The synthesis of vitamin $K_{2(20)}$ from menadione seems to go to completion if small physiological doses are given. This must be concluded from the fact that menadione has about the same activity in the vitamin K-deficient animal as have vitamin K_1 and K_2, compared on a molar basis (Section V, 2). However, the synthesis seems to be limited on administration of large doses. This is supported by the following findings.

As an antidote to Dicumarol, where larger doses are required, vitamin K_1 is much more active than menadione. Large doses of menadione, given to rats, are partly excreted as dihydromenadione diglucuronide or monosulfate. As described in Section VII, 1, labeled vitamin K_1 is stored in the liver to a much higher degree than labeled menadione.

VII. Metabolic Function

1. *Distribution of Vitamin K in the Animal Body and in the Cell Particulates*

A certain amount of vitamin K is stored in the animal body. After administration of C^{14}-labeled vitamin K_1 to rats, most of the radioactivity could be detected in the liver (Taylor *et al.*, 1956). The radioactivity after administration of labeled vitamin K_1 was twenty-four times higher than by administration of the same dose of labeled menadione (Taylor *et al.*, 1957). The observation that vitamin K_1 or K , given intravenously, had a much more prolonged therapeutic effect in correcting the proconvertin level than menadione, is probably also due to the better storage of the vitamins (Millar *et al.*, 1956).

Green *et al.* (1956) determined the vitamin K content of the liver cell particulates by the biological method, using vitamin K-deficient chicks. Of the total amount, 61% was found in the mitochondria fraction, 24% in the nucleus, and 15% in the supernatant, the calculation being based on the nitrogen content of the fractions. Considering the metabolic function of

vitamin K, it is interesting that vitamin K seems to be present in liver mitochondria in a similar amount as cytochrome c and riboflavin

As mentioned in Section VI, Martius (1956) has shown that menadione is transformed in the animal body into a vitamin K_2 compound with 20 C atoms in the isoprene side chain This was proved by the administration of C^{14} labeled menadiol diacetate to vitamin K deficient chicks In agreement with the results of Green *et al* (1956) on the vitamin K content of mitochondria, most of the activity was found in the mitochondria fraction of heart liver, and kidney, the highest being in that of heart

Bouman and Slater (1956) however could not find any appreciable amount of vitamin K in the Keilin and Hartree heart muscle preparation, using the biological test on vitamin K deficient chicks

2 The Role of Vitamin K in the Respiratory Chain Phosphorylation

In recent investigations Martius (1954) provides evidence that vitamin K_1 is involved in the biological oxidation system This theory is supported by the following findings The respiratory chain phosphorylation of liver mitochondria, obtained from vitamin K deficient chicks is depressed compared with that of liver from normal chicks (Martius and Nitz Litzow, 1954a) The *in vitro* addition of vitamin K_1 normalizes the oxidative phosphorylation (Martius and Nitz Litzow 1954b), whereas menadione and the vitamin K_1 analog with 5 C atoms in the side chain have no such property (Martius and Nitz Litzow, 1955) However, the activity of the analog with 15 C atoms in the side chain was only slightly less active than vitamin K_1 Anticoagulants such as Dicumarol the action of which could be antagonized by vitamin K_1, are potent uncouplers of oxidative phosphorylation (Martius and Nitz Litzow 1953)

With regard to the mode of action of vitamin K_1 in the respiratory chain, Martius (1954) assumed that vitamin K_1 is involved in the electron transferring system Considering two alternative pathways from DPNH to cytochrome c, in the one where diaphorase or cytochrome c reductase is an intermediate step no phosphorylation occurs while the other one involving vitamin K, is coupled with phosphorylation In the vitamin K deficiency state, only the first pathway is possible, this means that only two of the three phosphorylation steps coupled to the oxidation are in operation This is in accordance with the finding that the phosphorylation rate in liver mitochondria from vitamin K deficient chicks is reduced to about 70% (Martius and Nitz Litzow 1954a) The main arguments for the participation of vitamin K_1 in the electron transferring system are the occurrence of a highly active vitamin K reductase in the liver and the fact that Dicumarol and other vitamin K antagonists are potent competitive inhibitors (Martius and Strufe 1954, Martius, 1957)

This last point has been criticized by Colpa Boonstra and Slater (1958), who were not able to establish that vitamin K_1 is reduced to dihydrovitamin K_1 by this enzyme From experiments using menadione as a model substance, they concluded that this substance is not acting between DPNH and cytochrome b, but must enter the chain in the region of the flavoprotein or cytochrome b They stated, however, that there was a strong possibility of this reaction being entirely artificial

The finding of Cooper and Lehninger (1956) that Dicumarol also uncouples the oxidative phosphorylation linked to the oxidation of reduced cytochrome c is not in full agreement with the ideas of Martius and Nitz Litzow (1953, 1954b), that the uncoupling effect of Dicumarol is related only to the first steps, considered by them to be dependent on vitamin K_1 It must be supposed, therefore, that either the action of Dicumarol is not related specifically to vitamin K_1 or that vitamin K_1 is acting in a manner different to that assumed by Martius and Nitz Litzow (1954a,b) Cooper and Lehninger favor the role of vitamin K as a component of the phosphate transferring enzyme system at the three known phosphorylating sites in the respiratory chain

On the other hand, the investigations of Brodie and Gray (1955a,b), using bacterial enzymes, furnish evidence for the participation of vitamin K_1 in the electron transferring system These authors have combined a particulate and a supernatant fraction to get a phosphorylating system By fractionation of the supernatant, both its menadione reductase activity and its ability to restore phosphorylation are increased

Other investigations from these authors show that oxidation and phosphorylation are inactivated by ultraviolet light and that it is possible to restore both by addition of vitamin K_1 Menadione and vitamin K_2 were inactive (Brodie *et al*, 1957), while vitamin K_1 was more active than its analogs with 15 or 25 C atoms in the side chain (Brodie, 1958)

Similar results were obtained by Dallam and Anderson (1957) on rat liver mitochondria After inactivation by irradiation, the oxidative phosphorylation was reduced to a third of the initial value, while the oxidation was not impaired Addition of vitamin K_1 restores the phosphorylation From the observation that two thirds of the phosphorylation rate is dependent on vitamin K_1, they conclude that two of the three phosphorylation steps are concerned with vitamin K_1 On this point they do not agree with Martius and Nitz Litzow (1953, 1954a,b), who considered vitamin K to be involved in only one step As phosphorylation coupled with oxidation of reduced cytochrome c is not impaired by irradiation (Dallam and Anderson, 1958), vitamin K action seems to be related to the phosphorylating system of the chain between DPN and cytochrome c

Green *et al* (1955) could not find any reduction of the succinoxidase and

DPNH cytochrome c reductase activity in vitamin K deficient chicks and Dicumarolized rats They stated, however, that these findings are no argument against the speculations of Martius (1954) on the participation of vitamin K_1 in the electron transferring system

A criterion for a substance to be a member of the respiratory chain is its ability to reactivate an enzymatic system from which this component has been removed Such a system has been described by Nason and Lehman (1956), who used a rat skeletal muscle cytochrome c reductase inactivated by extraction with isooctane In such a test vitamin E (Nason and Lehman, 1956) and vitamin K (Deul *et al*, 1958) were found to be active In an extensive study Weber *et al* (1958a, b) showed, however, that this reactivation is not related to the redox system but to the isoprene like side chain Esterified tocopherol and the diacetates of the dihydro forms of vitamin K_1, K_2, and of ubiquinone(50) are as active as the free forms Moreover, isolated side chains, such as phytol and squalene, showed exactly the same activity as the corresponding menadione derivatives, compared on a molar basis The authors speculate that in this experiment a special function of the isoprene like side chain namely the binding of this vitamin to the fat material of the mitochondria, is demonstrated

3 Tentative Role of Vitamin K in Photosynthesis

Arnon *et al* (1955) have shown that the photosynthetic phosphorylation rate can be increased by addition of menadione phthiocol and other menadiones Similar results were obtained by Wessels (1955) As vitamin K_1 is widely spread in plant cells, these authors believe that vitamin K_1 may play a specific role in photosynthetic phosphorylation The tentative mechanism of the action of vitamin K_1 is discussed by Arnon *et al* (1958) and Marrè and Servettaz (1958)

4 Conclusions

Bouman and Slater (1957) have pointed out that, before a particular substance is accepted as a component of the main respiratory chain of enzymes the following criteria should be satisfied

(a) It is present in enzyme preparations in amounts commensurate with the enzymatic activity

(b) Its removal from the enzyme preparation results in inactivation of the enzyme and the activity can be specifically restored by addition of the substance

(c) It can be shown to undergo oxidation and reduction during the operation of the enzyme system at a rate in agreement with the over all enzymatic activity

Applying these criteria to the participation of vitamin K_1 in the respira

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The Role of Hepatic Glucose 6-phosphatase in the Regulation of Carbohydrate Metabolism

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I Introduction

Many attempts have been made to assign to enzymes the role of biochemical regulators as well as the function of catalysts For example, changes in the rate of glycogenolysis have been associated with changes in phosphorylase activity (Sutherland and Cori, 1951) Although the mechanism of hormone action is not completely understood, many instances have been recorded of change in enzymatic activity under various endocrine conditions (Knox *et al*, 1956, Miner and Henegan, 1951, Roberts and Szego, 1953) Congenital diseases involving abnormal metabolism have been attributed to an ever increasing number of enzyme deficiencies

Glucose 6 phosphatase, an enzyme involved in hepatic glucose production, has been studied from these several points of view Recent studies have indicated that this enzyme may have a role in the regulation of hepatic glucose production The activity of hepatic glucose 6 phosphatase is altered by fasting and alloxan diabetes, is virtually absent in glycogen storage disease and hepatoma, and is readily changed by endocrine manipulation Such studies have been summarized in this report for review and evaluation An extension of these observations has revealed a striking correlation between the activity of this enzyme and endocrine regulation of carbohydrate metabolism

In bringing together data from several laboratories, a comparison of results is made difficult by variations in methods of enzymatic assay and in the preparation of animals Therefore, in the presentation of data an attempt has been made to indicate variables which are of importance in the interpretation of experimental results Attention has been given to the following conditions age, weight, strain and species of animals, nutritional conditions (fed, fasted, length of fasting), method of sacrifice, and preparation of tissue for assay In relating the significance of an apparent change

in enzymatic activity to the whole animal it is of special importance to consider the basis of expression of enzymatic activity per unit tissue (wet weight, nitrogen, average cell), per organ or per 100 gm body weight

II Biochemistry of Glucose 6 phosphatase

1 *Identification*

The first studies made by Cori and Cori (1938) on hepatic glycogenolysis and hepatic glucose production suggested that the formation of glucose was mediated by a phosphatase Further study of liver glycogenolysis (Cori *et al* 1939, Ostern *et al*, 1939) showed that glucose release from liver was by way of hydrolysis of a hexose phosphate, but it was not clearly established whether glucose 1 phosphate (G 1 P) or glucose 6 phosphate (G 6 P) was the ester split In a reinvestigation of the problem of glucose release from hepatic tissue, Fantl and Rome (1945) incubated liver brei with Embden's equilibrium ester (G 6 P F 6 P) If a nonspecific phosphatase split this ester, glucose to fructose liberated would be expected to be in a ratio of 2 1 Instead, a ratio of 8 1 was found These authors therefore concluded that there was present in liver a specific G 6 Pase responsible for the release of glucose

Broh Kahn and Mirsky (1948) studied the relative rates of release of glucose from G 1 P and G 6 P by liver brei and from their data concluded that no specific glucose 1 phosphatase exists in liver and that G 1 P is converted to G 6 P before hydrolysis The report by Faulkner (1955) of a glucose 1 phosphatase in silkworm blood prompted a reinvestigation of this problem by Goodlad and Mills (1956) By differential centrifugation these authors were able to separate phosphoglucomutase and G 6 Pase activities and demonstrate a two step hydrolysis of G 1 P involving first conversion to the 6 phosphate ester and then hydrolysis

De Duve *et al* (1949) and Swanson (1949) have succeeded in partially purifying hepatic G 6 Pase and separating this activity from that of acid phosphatase The properties of this enzyme differ markedly from those of acid and alkaline phophatases and will be discussed in the following sections under the properties of G 6 Pase

2 *Assay Methods*

Glucose 6 phosphatase catalyzes the reaction glucose 6 phosphate → glucose + inorganic phosphate The activity of this enzyme can easily be assayed by measuring the appearance of inorganic phosphate following the incubation of G 6 P with liver homogenates (de Duve *et al*, 1949, Swanson, 1950) In the usual assay, homogenate representing 10–20 mg of wet liver is incubated with 0 2 *M* G 6-P in citrate buffer (pH 6 4) at 30° or 38° C

The reaction is terminated by the addition of trichloroacetic acid and inorganic phosphate measured by the method of Fiske and Subbarow (1925)

A manometric assay for G 6 Pase has also been devised (Ashmore *et al*, 1954) utilizing a coupled reaction between G 6 Pase and glucose oxidase This method depends upon the measurement of glucose released from G 6 P The glucose liberated is converted to gluconic acid by glucose oxidase in the presence of catalase, and the oxygen consumption is measured mano metrically

3 pH Optimum

Liver G 6-Pase is most active between pH 6 and 7 (Ashmore *et al*, 1954, Cori and Cori, 1952, de Duve *et al*, 1949, Swanson, 1950) Activity of this enzyme falls off rapidly on either side of 6 5, such that less than 20% of the optimum activity can be demonstrated at pH 5 or 9 In respect to pH optimum, this enzyme differs markedly from acid and alkaline phos phatases De Duve and Beaufay (1951) found that a rapid inactivation of G 6 Pase occurs below pH 5 5 and that the enzyme is precipitated at pH 5 0 By this method G 6 Pase activity can be separated from acid phos phatase Maximum stability of the enzyme is found at pH 8 0 (Beaufay and de Duve, 1954a)

4 Temperature Stability

Fantl and Rome (1945) noted that G 6 Pase activity was lost in liver homogenates stored for several days at +6° C However, liver microsome preparations rich in G 6 Pase activity may be kept for several days at 6° in Versene buffer without excessive loss of activity (Beaufay *et al*, 1954b) On the other hand, there is appreciable loss of activity in whole homogenates standing for only a few minutes at room temperature If liver tissue is frozen (Cori and Cori, 1952), there is no appreciable loss of G 6 Pase activ ity over a period of several months There is also no loss of G 6 Pase activ ity in rat liver slices incubated in oxygenated buffer for periods up to 90 minutes (Cahill *et al*, 1957a) However, activity is lost rapidly in anoxic liver at 38° C

5 Specificity

Inorganic phosphate is split from a number of organic phosphates when they are incubated with liver homogenate under conditions optimum for G 6 Pase Table I summarizes the results of such studies by Beaufay and de Duve (1954a), Crane (1955), Maley and Lardy (1956) Although a large number of organic phosphates are hydrolyzed by microsome prepara tions, all of the closely related hexose phosphates are hydrolyzed at one fifth or less the rate of G 6 P Beaufay and de Duve (1954a) conclude that

G 6 Pase is the enzyme catalyzing the hydrolysis of these substrates since activity is lost by briefly acidifying the preparation to pH 5 Under such conditions neither acid nor alkaline phosphatase activity is destroyed Evaluation of K_m for the various organic phosphates indicates that the ability of these substrates to inhibit competitively G 6 P hydrolysis is in accord with a single enzymatic activity (Crane, 1955)

TABLE I

RELATIVE RATES OF HYDROLYSIS OF VARIOUS ORGANIC PHOSPHATES BY LIVER MICROSOMES

Substrate	Rate	Reference
Glucose 6 phosphate	100	Crane (1955)
Allose 6 phosphate	90	Crane (1955)
1 5 Sorbitan 6 phosphate	74	Crane (1955)
Mannose 6 phosphate	50	Crane (1955)
Galactose 6 phosphate	28	Beaufay and de Duve (1954a) Crane (1955)
Ribose 5 phosphate	30	Beaufay and de Duve (1954a)
Phenyl phosphate	24	Beaufay and de Duve (1954a)
α Glycerol phosphate	20	Beaufay and de Duve (1954a)
Fructose 6 phosphate	19	Beaufay and de Duve (1954a)
2 Deoxyglucose 6 phosphate	20	Maley and Lardy (1956)
Tagatose 6 phosphate	11	Maley and Lardy (1956)
Glucosamine 6 phosphate	8	Maley and Lardy (1956)
Fructose 1 phosphate	7	Beaufay and de Duve (1954a)
β Glycerol phosphate	5	Beaufay and de Duve (1954a)
6 Phosphogluconate	4	Beaufay and de Duve (1954a)
N acetyl glucosamine 6 phosphate	2 2	Maley and Lardy (1956)
Fructose 1 6 diphosphate	1 4	Beaufay and de Duve (1954a)
Glucose 1 phosphate	0 5	Beaufay and de Duve (1954a) Maley and Lardy (1956)

The rate of hydrolysis of various organic phosphates by rat liver microsome preparations incubated at pH 6 7 expressed in relation to G 6 P hydrolysis which is arbitrarily expressed as 100

In whole homogenates the interconversion of F 6 P to G 6 P is so rapid that the inorganic phosphate released is accompanied by almost equivalent quantities of glucose (Beaufay *et al* 1954, de Duve 1953) However, washed liver microsomes free of phosphoglucose isomerase also split F 6 P the products in this case being fructose and inorganic phosphate In order to test the relative affinity of this enzyme for the two substrates, mixtures of G 6 P and F 6 P were incubated with washed microsome preparations and activity was followed manometrically (Ashmore *et al*, 1954) The rate of glucose release was not altered by addition of up to 4 moles excess F 6-P

6 Transphosphorylation

In addition to catalyzing the hydrolysis of glucose 6 phosphate, liver microsomes also catalyze an exchange of labeled glucose for unlabeled glucose in the hexose phosphate ester Hass and Byrne (personal communication) envision the reaction sequence as follows Enzyme + G 6 P = enzyme − G 6-P complex = enzyme − P + G Such a sequence of reactions would be consistent with the observed exchange of C^{14} between labeled glucose and unlabeled glucose 6 phosphate in the presence of liver microsome preparations, as well as with the competitive inhibition of hepatic glucose 6 phosphatase activity by glucose (Segal *et al*, 1958)

A number of closely related hexoses other than glucose have been found to inhibit glucose 6 phosphatase activity as measured by the release of P_i from G 6 P One of the most effective inhibitors is 3 deoxyglucose In this particular case it has been demonstrated that an exchange reaction occurs Glucose 6 phosphate + 3 deoxyglucose = 3 deoxyglucose 6 phosphate + glucose (M Nemer and J Ashmore, unpublished)

Exchange reactions of this sort lead to many interesting points of speculation For example, by such an exchange it is possible to introduce labeled glucose, or glucose derivatives as 3 deoxyglucose, into the glycolytic system without phosphorylation by a kinase and ATP It is also possible that inhibition of G 6 Pase activity by glucose serves in the regulation of hepatic glucose production

The enzymatic transfer of phosphate groups mediated by a number of tissue phosphatases has been more extensively reviewed by Axelrod (1956)

7 Activation and Inhibition

The instability of G 6 Pase to pH and temperature has been previously discussed Loss of G 6 Pase activity following dialysis has been noted (Fantl *et al*, 1945, Swanson, 1950) Activity is also destroyed by brief contact with acetone or ethanol at 0° and in concentrations as low as 10% (Swanson, 1950)

a Ions Neither calcium, magnesium (Swanson, 1950), nor sodium and potassium (Ashmore *et al*, 1954) alter G 6 Pase activity although slight inhibition by high concentration of calcium and magnesium has been observed (Beaufay and de Duve, 1954a) The enzyme is not inhibited by Be ion at 10^{-3} *M* (Ashmore *et al*, 1954, Cochran *et al*, 1951) in contrast with alkaline phosphatase, ATPase, and phosphoglucomutase, which are strongly inhibited by beryllium at this concentration Zinc, copper, and iodide ions result in irreversible inactivation of the enzyme (Beaufay *et al*, 1954) Glucose 6 phosphatase is strongly inhibited by 0 1 *M* fluoride, molybdate, and arsenate (Swanson, 1950)

b Phlorizin Although phlorizin has been used to inhibit various facets

of glucose metabolism over a period of over half a century the exact mechanism or mechanisms of its action are not well understood In relation to liver metabolism, Cori and Cori (1938) first observed that phlorizin inhibited the formation of glucose by liver homogenates Extension of these studies by Broh Kahn and Mirsky (1948) and by Fantl and Rome (1945) indicated that the phlorizin inhibition was in the hydrolysis of G 6 P to glucose and inorganic phosphate Although this would indicate phlorizin inhibition of hepatic G 6 Pase Swanson was unable to demonstrate any phlorizin inhibition of purified G 6 Pase preparations (Swanson, 1950) However, recent studies using crude homogenates of both liver and kidney indicate phlorizin inhibition of G 6 Pase activity in these tissues (Ashmore, unpublished) Inhibition analysis (Lineweaver Burk plots) of studies on kidney homogenates indicates that the inhibition is competitive Considering the possible role of G 6 Pase in glucose resorption from the kidney tubule and the mechanism of phlorizin glucosuria, this problem should be further investigated

c Bile Acids In attempts to separate G 6 Pase from other proteins, Beaufay *et al* (1954) have incubated liver microsomes with lecithinase, ribonuclease, and deoxycholic acid They observed that complete dissolution of the microsome by these substances resulted in a loss of G 6 Pase activity Bile acids over a range of 10^{-6} to 10^{-2} *M* may either markedly accelerate or inhibit the phosphatase reaction, depending upon the concentration of bile acid used (Ashmore *et al*, 1954) Concentration of deoxycholate up to 10^{-4} *M* and greater than 10^{-3} *M* inhibited the enzyme However between 10^{-4} *M* and 10^{-3} *M* a twofold increase in activity was observed Glycocholic and taurocholic acids were also observed to have this effect

d SH Reagents Although no inhibition is observed with iodoacetate (Swanson, 1950), both *N* ethylmaleimide and *p* chloromercuribenzoate at 0 001 *M* inhibit G 6 Pase (Ashmore, unpublished) Inactivation by copper and alloxan (Beaufay and de Duve 1954a) also suggests possible involvement of SH groups in the enzyme

A phosphatase inactivating system is found in the soluble fraction of liver (Beaufay *et al* 1954) Such inactivation may be inhibited by epinephrine in excess of the substrate and by chelating agents such as Versene The products of reaction tend to inhibit slightly In this respect phosphate has a more pronounced effect than does glucose

8 *Expression of Glucose-6 phosphatase Activity*

Since this enzyme is most frequently assayed by phosphate release, activity is usually expressed in terms of milligrams or micromoles of inorganic P liberated from G 6-P during incubation In studies on the physi

ology of G 6 Pase, various units of reference have been taken Cori and Cori (1952) have expressed activity per unit of wet weight or dry weight and per milligram nitrogen In animal studies, activity has been expressed as activity in total liver per unit of body weight and per average cell, as well as per unit of wet weight and per milligram nitrogen (Weber *et al*, 1956) In most cases in which phosphatase activity has been compared under differing sets of conditions, the results are the same regardless of the mode of expression of G 6 Pase However, in some conditions such as fasting and hormone treatment, marked changes in liver mass and in body weight may occur In such cases activity should be compared on some basis other than wet weight Activity per total liver or on a liver per 100 gm body weight ratio are perhaps most meaningful in considering the relation of an enzyme activity to the economy of the organism

III Cytochemistry of Glucose 6 phosphatase

1 *Distribution of Glucose 6 phosphatase in Animal Tissues*

a In Adult Animals The presence of G 6 Pase activity in liver tissue was demonstrated by early workers in this field (Fantl *et al*, 1942, Fantl and Rome, 1945) Systematic investigation of this enzyme in animal tissues has recently been conducted The data on G 6 Pase activity in various normal rat organs determined in extracts (de Duve *et al*, 1949 Hers and de Duve, 1950) were generally confirmed by results obtained in homogenates (Weber and Cantero 1955a) (see Table II) It is interesting that the G 6 Pase activity of the kidney (per gram wet weight and per milligram nitrogen) is as high as that of the liver (Weber and Cantero, 1955a) Possibly this high G 6 Pase activity is connected with the ability of the kidney to release sugar into the bloodstream (Drury *et al*, 1950, de Duve *et al*, 1955) Organs which do not release glucose into the bloodstream have little or no activity

b In Embryonic and Newborn Animals G 6 Pase activity in embryos has been examined only in the liver Nemeth (1954) studied G 6 Pase in the liver of the fetal *guinea pig* Pregnant guinea pigs were sacrificed by dislocation of the cervical vertebrae The enzyme was assayed in tissue homogenates made up in distilled water All reaction mixtures were 2% wet weight in tissue and 10 mmolar in G 6 P and were incubated at 35° C A buffer was not used, but the reaction mixtures were adjusted to pH 6 8 with a few drops of dilute HCl Under these conditions there was no G 6 Pase activity found in the fetal guinea pig liver until, or just before, term To rule out the possibility of a deficiency of a heat stable cofactor in the embryonic tissue, boiled homogenate prepared from adult guinea pig liver was added to a reaction mixture containing fresh embryonic liver No

effect was demonstrated It was also shown that the absence of activity in embryonic liver was not due to the presence of an inhibitor, because homogenates of fresh fetal liver did not inhibit a reaction mixture containing adult liver homogenate

The absence of G 6 Pase activity in fetal liver and the appearance of high activity in the liver of newborn animals was also demonstrated in the *rat* (Weber and Cantero, 1955b) In these studies G 6 Pase activity was measured in homogenates prepared in 0 25 *M* sucrose according to the method of Cori and Cori (1952), and the activity was expressed per unit wet weight of tissue

TABLE II*

GLUCOSE 6 PHOSPHATASE ACTIVITY IN NORMAL RAT ORGANS

Organ	Activity[b]	Percent	Specific activity[d]	Percent
Liver	213	100	71 0	100
Kidney	203	95	73 0	103
Spleen	16	8	5 4	8
Brain	11	5	4 9	7
Intestine	8	4	4 2	6
Lung	1	1	1 0	1
Muscle	1	1	1 0	1

From Weber and Cantero (1955)
The values are the mean of s[illegible] or more determinations
[b] Micrograms of phosphorus liberated in 15 minutes per 100 mg wet weight at 31 C
Liver arbitrarily assigned a value of 100%
[d] Micrograms of phosphorus liberated in 15 minutes per milligram nitrogen at 31 C

An attempt was also made to express enzymatic activity per average cell, and cell counts were made on embryonic and newborn rat liver These cell counts were extremely high, and it was recognized, on the basis of histological sections, that besides the parenchymal cells the different developmental forms of white and red blood cell series could interfere with correct counting Therefore the expression of G 6 Pase activity on per cell basis was not considered advisable in embryonic and newborn rat liver (Fig 1)

An attempt was made to correlate liver glycogen with G 6 Pase activity In the opinion of Nemeth (1954), the accumulation of liver glycogen in the late phase of gestation is due to the absence of G 6 Pase activity in this organ A parallelism was drawn with a certain type of glycogen storage disease which was considered by Nemeth (1954) as a persistence of the fetal state However, in studies in the human the presence of G 6 Pase was demonstrated in embryonic liver (Ashmore, unpublished) In all cases

appreciable activity was observed In the human, G 6 Pase appears quite early in the gestation period (fourth or fifth month)

2 Intracellular Distribution of Glucose 6 phosphatase

The localization of G 6 Pase in the cell has been examined by quantitative chemical determination of the enzyme in subcellular fractions and by histochemical study of tissue sections All studies on the intracellular localization of this enzyme have been performed in liver and kidney

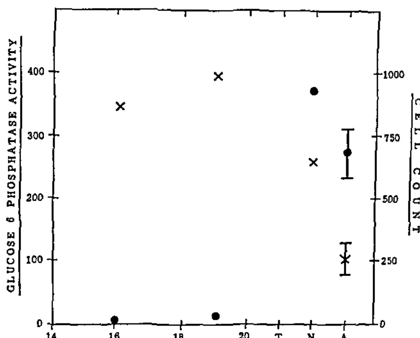


FIG 1 Glucose 6 phosphatase activity and cellularity in embryonic newborn and adult rat liver ● = G 6 Pase activity expressed in micrograms phosphorus liberated in 15 minutes at 31 C per 100 mg wet liver X = cell counts expressed in millions of nuclei per gram wet liver 14 16 18 20 = days of gestation, T = term N = newborn A = adult *Reprinted from Weber and Cantero (1955b)*

a Differential Centrifugation Studies The greatest contribution to the localization of G 6 Pase activity in the cell was given by the application of differential centrifugation methods By this approach, liver tissue of rats, mouse, guinea pig, frog, and rabbit were investigated The results of the different laboratories are not strictly comparable because of differences in species, sex, age, and nutritional condition of animals used in these studies However, a reasonable agreement is reached in recognizing the microsomal fraction as the main source of G 6 Pase activity (Appelmans *et al*, 1955, Berthet and de Duve, 1951, de Duve *et al*, 1955, Hers *et al*, 1951, Schneider *et al*, 1950, Swanson, 1949 Weber *et al*, 1956) It has also been shown that in the rat 24 hour fasting does not alter ellular distribution of

this enzyme (Weber *et al*, 1956) High doses of cortisone administration were without effect on the percentage distribution of G 6 Pase activity although the activity of the average cell was markedly increased as compared to that of a normal cell Fasting of cortisone treated animals for 24 hours also failed to influence the percentage of intracellular distribution of the enzyme (Weber *et al*, 1956) It is of interest to note that supernatant fluid prepared by centrifugation at 100 000 *g* for 30 minutes yields a liver extract free of G 6 Pase activity This may be of use in carbohydrate studies when the presence of G 6 Pase activity is undesirable

A further development in the localization of G 6 Pase activity may be expected when improved cell fractionation methods and electron microscopic check of the morphology of isolated fractions are applied in the study of this enzyme

b Histochemical Studies Chiquoine (1953) demonstrated G 6 Pase in frozen sections of mouse liver and kidney In both organs the enzyme is present only within parenchymal elements and is absent in stromal tissues It was shown that the enzyme was more abundant in the peripheral third of the hepatic lobule than in the inner two thirds Inside the liver cell G 6 Pase is concentrated about the nuclear membrane No significant changes in enzyme distribution in the liver were observed in relation to the diurnal cycle

In the kidney the enzyme was demonstrated in the proximal convoluted tubule, concentrated at the basal pole of the cells and in portions of the renal glomerulus Deane (1944) reported that the basophilic bodies in the hepatic cells of white mice are usually grouped about the nucleus and has equated these bodies with the microsomal fraction of differential centrifugation studies Since most investigators have shown that G 6 Pase is associated with the microsomal fraction of liver cells (Table III) the histochemical distribution of the enzyme as reported by Chiquoine is consistent with Deane s description of the distribution of the microsome material

The histochemical demonstration that the distribution of the enzyme does not change significantly during a 24 hour period after feeding is in line with the report (Weber *et al*, 1956) based on chemical determination that G 6 Pase activity is maintained at normal level in the *average liver cell* after 24 hour fasting

On the basis of the specific biochemical characteristics of G 6 Pase, it was possible to show that this enzyme is located in cellular regions different from those in which acid or alkaline phosphatases are found (Chiquoine, 1953) Similar histochemical procedures were used recently in studying the seminal vesicles and prostate gland of the rat (Kuhlman 1954) It was shown that tissues of the male accessory glands are capable of hydrolyzing both G 6 P and F 6 P The histochemical reaction demonstrated in this

TABLE III*

INTRACELLULAR DISTRIBUTION STUDIES OF LIVER G 6 PASE†

Reference	Species strain and sex	Preparation of animals	Reaction mixture	Basis of expression of activity	Distribution* (%)	Recovery (%)	Footr
Swanson (1950)	Rat, albino	Fed	G 6 P, citrate buffer pH 6 5, 37 C	Wet weight	About 70% in impure Mc preparation	90	1,
Hers *et al* (1951)	Rat guinea pig rabbit	Fasted for 12 hours	G 6 P ABC buffer, pH 6 5, 38 C	Wet weight (%)	Rat G pig Rabbit *N*, *Mt* 5, 5 18 *Mc* 88, 82 85 *S* 11 8, 3	Rat 104 G pig 96 Rabbit 106	3
Berthet *et al* (1951)	Rat	Fasted for 12 hours	G 6 P ABC buffer pH 6 5 38 C	Wet weight (%)	*N* 6 *Mt* 13 *Mc* 75 *S* 6	100	
Kuff and Schneider (1954)	3 month old female mice 3CH		G 6 P, sodium succinate buffer pH 6 6 37 C	Wet weight, nitrogen	Only mitochondrial fraction was studied	83	4
Appelmans *et al* (1955)	Rat	Fasted for 12 hours	G 6 P cacodylate buffer pH 6 5 37 C	Wet weight, nitrogen (%)	*N* 10 7 *Mt* 7 4 *Mc* 79 3 *S* 2 3	99 7	5
de Duve *et al* (1955)	Rat adult albino	Fasted for 12 hours	G 6 P histidine and Versene pH 6 5 37 C	Wet weight (%)	*N* 6 8 *Mt* 9 7 *Mc* 73 8 *S* 2 7	92 8 ± 8 7	6

Sonnenschein *et al* (1955)	Frog male and female		C 6 P veronal buffer pH 7 6 37 C	Nitrogen	Only homogenate and *Mc* fraction were compared	Little activity in *Mc* fraction	7
Langdon *et al*	Rat young *male* Sprague Dawley 75–200 gm	Fed	G 6 P citrate buffer pH 6 5 30 5 C	Wet weight per 100 gm animal	65% in *Mc* fraction		Not reported
Weber *et al* (1956)	Rat male Wistar 100 ± 10 gm	Groups of fed and fasted for 24 hours	G 6 P citrate buffer pH 6 8 31 C	Wet weight nitrogen per average cell (%)	Normal untreated animals Fed: *N* 18, *Mt* 23, *Mc* 45, S 0 Fasted: 17, 27, 49, 0	Normal untreated animals Fed: 86 ± 13 Fasted: 93 ± 2	
					Cortisone injected animals Fed: *N* 22, *Mt* 34, *Mc* 45, *S* 0 Fasted: 22, 37, 39, 0	Cortisone injected animals Fed: 101 ± 5 Fasted: 98 ± 11	

From Ashmore *et al* (1956b)

† Abbreviations for the names of subcellular fractions *N* = nuclei *Mt* = mitochondria *Mc* = microsomes *S* = supernatant fluid

1 If not otherwise stated homogenates were prepared in 0 25 *M* sucrose

2 Homogenates prepared in 0 9% NaCl or 30% sucrose

3 0 25 *M* sucrose contained 3 × 10^{-4} *M* $KHCO_3$ General conclusion G 6 Pase is localized in the *microsomes* *The presence of this enzyme in other fractions is due to contamination*

4 Relative concentrations of G 6-Pase activity paralleled those of PNA P almost exactly in all isolated fractions of mitochondria Fractions were examined by phase microscopy

5 *Fractions were examined by phase microscopy*

6 0 5 *M* sucrose contained 0 001 *M* Versene

7 Kassel's solution was used instead of 0 5 *M* sucrose This special electrolyte medium used for fractionation may be the cause of the lack of activity in *Mc* fraction

study permitted detection of a labile enzyme localized differently from the more stable acid and alkaline phosphatases The epithelia of the seminal vesicles and prostate gland showed evidence of phosphatase activity in the cells when either F-6-P or G 6-P were used as substrates The connective tissue stroma beneath the epithelium was negative The optimum pH for the reaction was between 6 3 and 7 0 The intensity of the reaction fell off rapidly above and below these values and was zero at pH 5 and 10

IV Physiology of Glucose 6 phosphatase

1 *Relation to Blood Glucose*

Since the maintenance of the blood glucose, arising endogenously, is de pendent upon the presence of the liver and more specifically upon adequate glucose 6 phosphatase activity in the liver, it is but logical to suspect that this enzyme might be involved in the regulation of hepatic glucose produc tion Two types of studies have been done in attempt to relate G 6 Pase activity to glucose production (1) comparison of G 6 Pase activity and blood glucose in fasting animals, (2) comparison of G 6 Pase activity and *in vitro* glucose production in rat liver slices

Blood glucose is regulated by four factors (a) rate of absorption of dietary glucose, (b) hepatic glucose production, (c) peripheral utilization, and (d) in extreme cases, by renal excretion In fasting rats the contribu tion of intestinal absorption is eliminated and, in the absence of specific peripheral inhibition, utilization by extrahepatic tissues should remain rather constant Under such conditions a positive correlation between blood glucose levels and hepatic G 6 Pase activity can be observed in fasting normal, cortisone treated, adrenalectomized and hypophysecto mized rats (Weber and Cantero, 1955c, 1957c) and in obese hyperglycemic mice (Shull *et al*, 1956) This is illustrated by Fig 2 It should be noted, however, that the demonstration of a correlation between G 6 Pase activity and blood glucose depends to some extent on the basis upon which G 6 Pase activity is expressed Since the enzyme is concentrated mainly in the liver, expressing total phosphatase activity per 100 gm body weight is expected to give information about the "total available" G 6 Pase activity in the organism When this is done a parallelism between total liver G 6 Pase activity and blood sugar level may be observed (Fig 2)

Glucose production by rat liver slices obtained from normal, diabetic, adrenalectomized diabetic, and adrenalectomized normal rats shows some correlation with the G 6 Pase activity of the tissue (Table IV) (Ashmore *et al*, 1956b) However, in time sequence studies, it can be demonstrated that the increased glucose production by the tissue *in vitro* precedes an increase in the phosphatase activity in the adrenalectomized diabetic rat

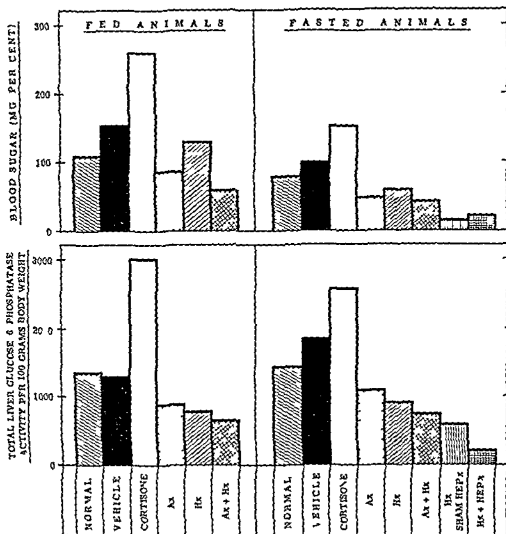


FIG 2 Comparison of blood sugar and hepatic glucose 6 phosphatase activity in the rat The blood sugar is expressed in milligrams per cent The enzymatic activity is expressed as total available liver G 6 Pase activity Normal untreated animals Cortisone animals were given daily intramuscular injections of 25 mg of cortisone acetate for 5 days and were sacrificed on the sixth day Vehicle animals injected the same way with the vehicle used as the suspending medium for cortisone *Ax* adrenalectomized rats *Hx* hypophysectomized rats *Ax* + *Hx* adrenalectomized and hypophysectomized rats Hx + SHAM HEPx hypophysectomized rats which were subjected to sham hepatectomy operation Hx + HEPx hypophysectomized animals which were subjected to partial hepatectomy Reprinted from Weber and Cantero (1957c)

treated with hydrocortisone Also, it should be noted that hepatic glucose production can be increased *in vitro* by glucagon or epinephrine or by alteration of the ionic composition of the incubation medium without any apparent increase in G 6 Pase activity (Cahill *et al* , 1957a) In these cases, liver phosphorylase is the enzyme whose activity is increased and the enhanced glucose released is the result of glycogenolysis Decreased glucose production of liver slices in the presence of phlorizin or fluoride (Niemeyer, 1955) may be the result of decreased G 6 Pase activity There is no question but what an absence of this enzyme will result in no glucose production, as is the case in von Gierke's disease, where there is a congenital absence of G 6 Pase (Cori and Cori, 1952)

TABLE IV

COMPARISON OF GLUCOSE PRODUCTION AND HEPATIC GLUCOSE 6 PHOSPHATASE ACTIVITY

	Glucose produced	*G 6 Pase* (μmoles P/gm liver)
Normal	69	151
Diabetic	149	375
Adrenalectomized diabetic	76	195
Adrenalectomized normal	67	178

A comparison of glucose production by rat liver slices incubated in a high K medium with hepatic glucose 6 phosphatase activity Glucose production is expressed as micromols glucose per gram wet liver per 90 minutes Glucose 6 phosphatase activity is expressed as micromoles P liberated per gram wet liver per 30 minutes

2 Relation to Glycogen Synthesis

One can easily imagine glycogen as an overflow reservoir for the G 6 P pool Under such conditions phosphatase would serve as the main drain of the pool and in this capacity might regulate the distribution of G 6 P between glucose and glycogen In abnormal conditions where there is a virtual absence of G 6 Pase in the liver, glycogen tends to accumulate An interesting relation between glycogen content and G 6 Pase activity in fetal liver has been noted in the preceding section (Nemeth, 1954, Weber and Cantero, 1955b)

In rat liver slices a relation between G 6 Pase and the percentage of G 6 P which goes to glycogen can be demonstrated (Ashmore *et al* , 1956b) (Table V) The percentage of G 6 P to glycogen has been experimentally derived from studies with labeled pyruvate and represents the ratio pyruvate converted to glycogen over pyruvate converted to glucose plus glycogen In these studies the percentage of G 6 P to glycogen decreased as the G 6 Pase activity increased It is therefore not unreasonable to

expect that G 6 Pase activity could regulate the relative percentage of G 6 P molecules converted to glucose and glycogen under specific condition of constant G 6 P production and in the absence of other enzymatic defects between G 6 P and glycogen

3 Rate of Glucose 6 phosphatase Relative to Other Pathways

In assessing the role of G 6 Pase in hepatic metabolism it is of importance to know the rate of the phosphatase pathway in relation to the other available pathways of G 6 P metabolism In liver four pathways of metabolism are available for G 6 P (1) to glycogen via phosphoglucomutase, (2)

TABLE V

RELATION OF LIVER GLUCOSE 6 PHOSPHATASE TO GLYCOGEN FORMATION

Rat	Time (hours)	Per cent G 6 P to glycogen	Glucose 6 phosphatase
Normal		50	157
Diabetic		12	354
Adrenalectomized diabetic		35	150
Adrenalectomized normal		47	178
Adrenalectomized diabetic + 17 hydroxycorticosterone	2	24	155
	6	23	162
	12	17	240
	24	10	246
	48	15	310

Glucose 6 phosphatase activity is expressed as micromoles of G 6 P split per gram of liver per 30 minutes Per cent G 6 P to glycogen is the ratio pyruvate to glycogen over pyruvate to glucose plus glycogen measured in rat liver slices during a 90-minute incubation

to glucose and inorganic phosphate via glucose 6 phosphatase, (3) to pentose and CO via the hexosephosphate oxidation pathway, and (4) fructose 6 phosphate (F 6 P) and the Embden Meyerhof glycolytic pathway via phosphohexoseisomerase

A comparison of the four enzymes initially competing for G 6 P shows that the equilibrium of both phosphoglucomutase and phosphohexoseisomerase favors G 6 P formation on the other hand G 6 P dehydrogenase and glucose 6 phosphatase result in G 6 P removal Assays of phosphatase and dehydrogenase activity in the same homogenate under conditions optimum for each enzyme indicate that the phosphatase is many times more active than the dehydrogenase (Ashmore *et al*, 1957b Glock *et al*, 1956, Perske *et al* 1957, Weber and Cantero 1957e) Measurement of the activities of phosphatase dehydrogenase mutase and isomerase (Weber and Cantero, 1957e) from the same liver homogenate showed that under conditions of

maximal enzymatic activity the relative rates of these four enzymes are phosphohexoseisomerase > phosphoglucomutase > G 6 Pase > G 6 P dehydrogenase (see Table VIII, Section VI)

From studies with rat liver slices and C^{14} labeled glucose and fructose one can calculate the relative rates of the four pathways of G 6 P metabolism Per 100 molecules of glucose phosphorylated, 2 are oxidized via the phosphogluconate oxidation pathway, 18 to glycogen, 55 are hydrolyzed by G 6 Pase and 25 are metabolized via the Embden Meyerhof glycolytic pathway Although these values are obtained from *in vitro* experiments, they strongly support the role of G 6 Pase in the regulation of hepatic glucose metabolism (Ashmore *et al*, 1957b)

V Regulation of Glucose 6 phosphatase Activity

1 *Introductory Remarks*

Regulation of an enzymatic activity in the sense of a direct interaction between the regulatory substance and the enzyme has not been demonstrated However, changes in the activities of many enzymes have been associated with over all metabolic regulation, particularly with respect to the endocrine hormones In the specific case of glucose 6 phosphatase, where the physiological function of the enzyme is known, it is often easy to interpret the results of hormonal studies in relation to the known general metabolic effects of the endocrine function under study Yet, a change of enzymatic activity following endocrine manipulation should in no case be construed to prove activation or inhibition of the enzyme by the hormone per se Indeed, in no instance has change in activity in glucose 6 phosphatase been noted other than after the *in vivo* administration of the regulatory substance over a period of several hours or several days

In addition to regulation of glucose 6 phosphatase activity by hormonal substances certain dietary alterations have been shown to cause marked changes in the activity of this enzyme This might suggest to some that hormones affect regulation through over all changes in intermediary metabolism, causing an increase in enzyme activity resulting from changes in substrate concentration Although such speculation is interesting, it remains at present without any experimental substantiation

An attempt is made in this section to present the few established facts relating to the hormonal and dietary regulation of liver G 6 Pase activity

It should be noted that in instances of reported changes in G 6 Pase activity resulting from hormone administration or removal, special experimental conditions may be necessary to obtain clear cut effects (Weber and Cantero, 1957c) (see Fig 2) The basis on which [illegible] natic activity is expressed may be of great im[illegible]ance, becau[illegible] [illegible]ssible change in liver weight and in liver [illegible] [illegible]t ratio [illegible] [illegible]tal circum

stances (Weber and Cantero, 1957c) However, it is most encouraging that the results obtained by the several laboratories reporting hormonal studies are in general agreement and do not require resolution of differences due to basis of calculation or specific technique employed

2 Adrenal Gland

a Hormone Administration Studies Administration of large doses of cortisone (25 mg per 100 gm body weight) to intact rats resulted in a marked increase in liver G 6 Pase activity (This increase was evident whether activity was expressed per gram wet liver, per milligram liver nitrogen, per average cell, or on a liver body weight ratio) Animals showing an increased hepatic G 6 Pase activity also exhibited elevated blood sugar levels and marked liver glycogen deposition (Weber *et al* , 1955) Administration of smaller doses of cortisone (1 5 mg per 100 gm body weight) (Menon and Gornall 1956) as well as hydrocortisone (Ashmore *et al* , 1954) also result in increased hepatic glucose 6 phosphatase in normal *fed* rats Hydrocortisone (5 mg per 12 hours) injected over a period of 48 hours will give an increase in G 6 Pase activity of 34% when activity is expressed per gram wet liver In only one instance has a failure to obtain an increase in hepatic glucose 6 phosphatase activity been reported after the administration of cortisone or adrenal cortical extracts (Langdon and Weakley, 1955)

An increase in G 6 Pase activity in animals fasted 24 hours before sacrifice can be obtained with cortisone administration (Weber *et al* 1956) (see Fig 2) The increased G 6 Pase activity of the homogenate of livers from cortisone injected rats was reflected in the increase of the enzyme activity in all three particulate fractions and was mainly due to increases in the G 6 Pase activities in the nuclear and mitochondrial fractions The possibility of a contamination of the nuclear and mitochondrial portions by microsomes was not completely excluded The control animals in this series were injected with the vehicle, which contained only the suspending medium without cortisone The administration of vehicle did not cause any significant alterations in the percentage distribution of G 6 Pase as compared to normal uninjected fed or fasted animals Cortisone caused an increase in the mitochondrial activity both in fed and fasted animals of 89% and 42% respectively From these results the following suggestion was made "The demonstration of the effect of cortisone on liver G 6 Pase activity brings up the possibility that the adrenal cortex may play a role in the physiological maintenance and regulation of this enzyme (Weber *et al* 1956)

Injection of hydrocortisone (1 mg per day) in hypophysectomized animals caused a definite increase in liver G 6 Pase activity (Harper, 1956)

b Hormone Removal Studies Effect of adrenalectomy in normal animals

The effect of adrenalectomy was examined on blood sugar and liver G 6 Pase activity in Wistar rats Enzymatic activity was expressed per average liver cell and per 100 gm body weight The results demonstrated that in *fasted* adrenalectomized animals the decreased blood sugar levels followed decreased liver G 6 Pase activities (Weber and Cantero, 1955c) Later it was reported that adrenalectomy in fed rats had no effect on liver G 6 Pase as expressed per gram wet liver or per total liver weight (Ashmore *et al*, 1956b)

In further studies (Weber and Cantero, 1957c) it was confirmed that liver G 6 Pase activity was apparently not affected in *fed* adrenalectomized rats when the data were expressed per unit wet weight, however, a significant decrease (32%) was found when data were expressed per average cell, or per 100 gm body weight (total available activity) In *fasted* adrenalectomized animals the liver G 6 Pase activity was decreased per wet weight, per cell, or per 100 gm body weight as compared to normal fasted animals The authors pointed out that in normal fasting animals the total available liver G 6 Pase activity is increased, while other enzymes involved in G 6 P utilization are markedly decreased (Weber and Cantero, 1956a, 1957d) On the other hand, in the absence of the adrenal gland or hypophysis the fasting animal is unable to increase liver G 6 Pase activity to normal fasting levels (Weber and Cantero, 1957c) (see Fig 2) However, the administration of cortisone over a period of 48 hours prior to fast restores the hepatic response to fasting to normal (Froesch *et al*, 1958)

Effect on adrenalectomy in diabetic animals Adrenalectomy in alloxan diabetics rats returned liver G 6 Pase activity to normal That this effect was due to the absence of adrenal cortical hormone was evidenced by the increase in G 6 Pase in the adrenalectomized diabetic rats to diabetic levels, 218% of normal, by injection of 17 hydroxycorticosterone (5 mg per 12 hours) over a 48 hour period (Ashmore *et al*, 1956b) It was also shown that in adrenalectomized animals insulin injection caused a more marked drop in liver G 6 Pase activity than in normal animals (Ashmore *et al* 1956b)

Effect of adrenalectomy in hypophysectomized animals When hypophysectomized rats were subjected to adrenalectomy, no significant additional reduction was found in fed or fasted animals (Weber and Cantero, 1957c)

c Mechanism of Action The following possibilities are considered for the mechanism of action of a hormone (1) direct effect on enzyme or enzyme synthesis (2) influence of enzymatic activity through cofactors, (3) indirect influence of enzymatic activity through substrate level (G 6 P)

Study of direct effect, in vitro effect of cortisone It was shown that the G 6 Pase activity of cell free liver homogenates was not influenced by the addition of cortisone even in high concentration Preincubation (15–60 minutes)

of liver homogenate with cortisone at 37° C also failed to affect G 6 Pase activity (Weber *et al* 1956)

Study of indirect effect Long term studies have brought support to the possibility that cortisone may act through influencing gluconeogenesis, which in turn would affect the G 6 Pase level in the liver cell (Ashmore *et al*, 1956b) These workers have demonstrated that in the biological sequence after cortisone administration to adrenalectomized diabetic rats the resulting increased gluconeogenesis preceded by several hours the increase in hepatic G 6 Pase Thus, it seems unlikely that the increased glucose production is a direct result of the effect of the hormone on the enzyme in the case of the diabetic animal On the other hand, it is possible that relations are different in the *normal* animals, and the possibility of a direct effect of hormones on liver G 6 Pase should be further explored

3 Pituitary Gland

a Hormone Administration Studies Effect of growth hormone Langdon and Weakley (1955) studied the effect of pituitary growth hormone (i p in a single dose 10 mg per kilo, 18 hours prior to sacrifice) on *fed* Sprague Dawley rats weighing 75–200 gm Liver G 6 Pase activity was expressed per gram of liver and per 100 gm body weight No significant difference was found in the G 6 Pase activity of homogenate and microsomal fraction of normal and growth hormone injected rats On the other hand, Menon and Gornall (1956) found that administration of 100 μg of growth hormone per 100 gm body weight almost doubled liver G 6 Pase in *normal* rats Growth hormone administration also restored hepatic G 6 Pase of *hypophysectomized* or *adrenalectomized* rats to normal levels

Effect of corticotropin (ACTH) Shull *et al* (1956b) demonstrated the effect of ACTH on liver G 6 Pase in male mice of LAF_1 strain The experiments were performed on mice grafted with radiation induced (Furth *et al*, 1953) ACTH secreting tumors One half of the animals at 10 weeks of age were grafted subcutaneously with the tumors The untreated mice served as controls All animals had food and water ad libitum The water contained animal formula Terramycin HCl at a level of 8 gm per gallon and served to prevent infection to which ACTH secreting tumor bearing mice are highly susceptible At 17 weeks of age all animals were sacrificed The tumors at this time averaged 1 cm in diameter the body weight of tumor bearing and control mice was about the same A modification of the assay of Swanson (1950) was used to measure liver G 6 Pase activity (Shull *et al*, 1956b) Enzymatic activity was expressed per gram wet weight and per total liver

The liver G 6 Pase activity per gram wet weight and per total liver was significantly higher in the tumor bearing mice than that of the normal mice

In addition, fasting did not cause a reduction in blood glucose level in the tumor bearing group, while it did in the normal controls Similarly, the tumor-bearing mice retained approximately four times more liver glycogen after 24 hour fast than their controls

Shull *et al* (1956b) draw the conclusion that "the striking increase in G 6 Pase activity per gm of liver observed in tumor-bearing mice indicates that the activity of this enzyme *in vivo* is probably controlled, at least in part, by the adrenal cortical hormones " The authors also noted that these tumor bearing mice excrete 0 5 μg per day total neutral C_{21} steroids in the urine of which corticosterone comprised 0 3 μg (Schull *et al*, 1956b) Only traces of Compounds E and F and no aldosterone were found Therefore, the 45% increase in G 6 Pase activity observed in the tumor bearing mice was probably produced largely by corticosterone

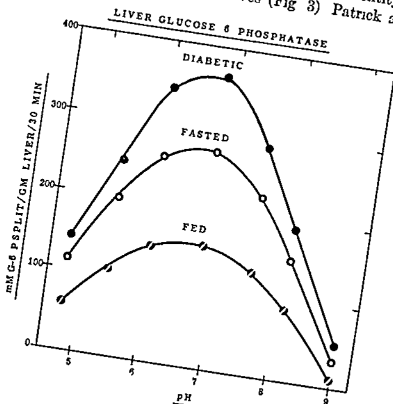
b Hormone Removal Studies Effect of hypophysectomy The effect of hypophysectomy on liver G 6 Pase activity was shown in Wistar rats of 100 gm weight (Weber and Cantero, 1957c), the blood glucose level was assayed and homogenate G 6 Pase activity was expressed per average liver cell and per 100 gm body weight In 24 hour fasted hypophysectomized animals the significantly decreased blood sugar levels were paralleled by decreased liver G 6 Pase activities When hypophysectomized animals were partially hepatectomized, 24 hour fasting resulted in hypoglycemic blood sugar levels and extremely low G 6 Pase activities (Weber and Cantero, 1957c) The lack of maintenance of blood sugar level and liver G 6 Pase activity in hypophysectomized animals when subjected to fasting is in marked contrast to the maintenance of blood sugar level and liver G 6 Pase activity in intact normal fasted rats (see Fig 2)

Harper described a fall in liver G 6 Pase activity (from about 4 5 to about 2 5 mg P/gm fresh liver per hour) after hypophysectomy, and similar low values were obtained in alloxan diabetic rats that had been hypophysectomized When rats were fed ad libitum no response to growth hormone injection was detected Combined injections of hydrocortisone and thyroxine increased the liver G 6 Pase activity of hypophysectomized rats to a level above that of the intact normal rat Injections of hydrocortisone or thyroxine separately did not restore normal activity (Harper, 1956) A decrease in hepatic G 6 Pase activity in hypophysectomized rats has also been described by Reid *et al* (1956)

4 Pancreas

a Hormone Removal Studies Effect on alloxan Since there is an increase in hepatic glucose production in alloxan diabetic rats, it might be expected that such animals would show an increase in liver G 6 Pase activity Phosphatase activity in the diabetic rats is found to be increased whether ex

pressed per gram wet liver, total liver, or per milligram nitrogen (Ashmore *et al*, 1954, Langdon and Weakley, 1955, Parks, 1954) The increase in activity in the diabetic has been observed both in whole homogenates and the microsomal fraction of liver Further evidence that a specific phosphatase activity is increased in the alloxan animal is the identity of the pH optima shown by the pH activity curves (Fig 3) Patrick and Tulloch



and microsome preparations, but comparable activation is observ similar preparations of liver from alloxan diabetic rats (Ashmore an bett, 1955) It therefore appears likely that the increase in activity ob in diabetes represents an actual increase in the enzyme

Studies by Segal *et al* (1958) on kinetic parameters of liver G 6 P normal and diabetic rats indicate that the apparent Michaelis co (K_m) for this enzyme is different in homogenates of liver from norm diabetic rats The average K_m found for G 6 Pase in normal liver w $\times 10^{-3} M$ while the K_m for the diabetic was 3 27 $\times 10^{-3} M$ The K served in homogenates of normal rat liver agrees with the average 2 $\times 10^{-3} M$ (Hass and Byrne, personal communication, Nemer, H and Ashmore, unpublished) In view of the differences in apparent K served by various investigators, using slightly different but still unpurified tissue preparations, the differences observed between n and diabetic tissues are difficult to interpret Such differences mig tually represent intrinsic differences in property which is retained b purified enzyme, but most likely they represent a slightly altered mi the diabetic liver Since G 6 Pase activity is found in the microsoma tion of liver cells, it is possible that changes in the composition of this tion could account for differences in kinetic parameters measured

Fasting in the alloxan diabetic rat results in a net decrease in tota G 6 Pase, although activity of the enzyme per gram of liver is uncha Fasted normal and alloxan diabetic rats therefore have about the same Pase activity per gram of liver (Ashmore *et al*, 1954)

Adrenalectomized diabetic rats have normal liver G 6 Pase ac (Ashmore *et al*, 1956b) The role of the adrenal steroids in regulation 6 Pase activity in the alloxanized rat has been discussed under the ad gland

b Biological Significance of Increased Liver G 6 Pase Activity in dia

What is the significance of the increase in G 6 Pase observed in diab An increase in the activity of several other enzymes has been note livers of alloxan diabetic rats (Ashmore *et al*, 1956b) Copenhaver (1951) have observed an increase in succinic oxidase, an increase in β curonidase (another microsomal enzyme) has also been noted (Tuba, 1 On the other hand, a decrease in G 6 Pase dehydrogenase has been rep (Glock and McLean, 1955) At present any relation between any of t various enzymatic activities is too remote to permit speculation

Not only can an increase in G 6 Pase cause increased glucose produc but it can also, by draining off the product of the glucokinase reac give the net result of diminished hepatic glucose utilization In order ther to elaborate the role of this enzyme in such possible reactions, a s of studies on the biochemical sequence of events was undertaken in all

diabetic rats treated with insulin and in adrenalectomized diabetic rats, with cortisone

c Hormone Administration Studies Effect of insulin and glucagon It was demonstrated in Wistar rats that injection of insulin into alloxan diabetic rats (5 units of protamine zinc insulin at 12 hour intervals beginning 48 hours prior to sacrifice of the animals) returned G 6 Pase activity per gram of liver toward normal (Ashmore *et al*, 1954) This finding was later confirmed by demonstrating that insulin administration (200 units of protamine zinc insulin per kilo injected subcutaneously 12 hours prior to sacrifice) lowered the activity of liver G 6 Pase of diabetic rats to nearly normal values (Langdon and Weakley, 1955)

The biological sequence of events was investigated in alloxan diabetic rats sacrificed at various times after insulin administration (Ashmore *et al*, 1956b) Fall of blood sugar in the animal, incorporation of carbon from pyruvate *2* C^{14} into glucose, and changes in hepatic G 6 Pase were compared There was an immediate response of blood sugar to injected insulin, normal values being reached in 5 hours Gluconeogenesis (conversion of pyruvate to glucose) continued at diabetic rates during this period and was returned to normal only after 24–48 hours of insulin administration The G 6 Pase activity, expressed per gram liver, likewise remained at diabetic levels for 6 hours after injection of insulin but had decreased after 12 hours

However, when hydrocortisone was administered to adrenalectomized diabetic rats, an increase in gluconeogenesis preceded by several hours any increase in G 6 Pase It has therefore been concluded that the increased G 6 Pase activity in the diabetic is the result of an increased gluconeogenesis (Ashmore *et al*, 1956b) This conclusion is further strengthened by the observation (Harper 1956) that rats maintained on a high protein diet have hepatic G 6 Pase activity comparable to that of the diabetic It is also apparent that the lack of insulin per se is not the cause of the increased phosphatase activity, since adrenalectomy of the alloxan diabetic returns the activity of this enzyme to normal (Ashmore *et al* 1956b)

The effect of an increase in G 6 Pase activity on the relative rates of the various pathways of G 6 P metabolism has been calculated, and it was found that in the normal liver *in vitro* 55% of the G 6 P is metabolized via the phosphatase reaction while in the diabetic this is increased to 73% Not only does the increased G 6 Pase activity result in increased hydrolysis of G 6 P to glucose, but also in a reduction of glycogen formation from 18% in the normal to 2% in the diabetic The data indicate that an *increase* in G 6 Pase does not markedly alter the relative rates of metabolism of G 6 P via the Embden Meyerhof and the phosphogluconate oxidation pathways

The effect of insulin *in vitro* was tested by adding the hormone to liver homogenates, however, no change was demonstrated in G 6 Pase activity

Attempts to demonstrate hormonal regulation of G 6 Pase *in vitro* have been regularly unsuccessful (Langdon and Weakley, 1955, G Weber, unpublished)

Injections of Glucagon (100 μg per 100 gm body weight) almost doubled liver G 6 Pase in normal rats (Menon and Gornall, 1956) Acute injection of glucagon in dogs under pentothal anesthesia, with serial liver biopsy over a period of 6 hours, caused an increase in G 6 Pase on wet weight basis However, the liver of dogs receiving the sustained glucagon infusion became perceptibly smaller during the experiment Thus, the apparent elevation of G 6 Pase activity may be only a concentration of the enzyme due to loss of glycogen and water and reduction of liver mass (Cahill *et al*, 1957b)

5 *Thyroid Gland*

Hormone administration studies An increase in hepatic G 6 Pase has been observed in animals made thyrotoxic by the injection of thyroxine over a period of several days (Glock *et al*, 1956, Harper, 1956, Maley and Lardy, 1955) Treatment of rats with 0 5 gm DL thyroxine per day for 8 days produced a 47 % increase in G 6 Pase activity per gram wet liver and 30 % of normal when expressed per total liver

Injection of thyroxine (5 gm per 100 gm body weight) is also effective in increasing liver G 6 Pase of hypophysectomized rats above normal (Harper, 1956, Menon and Gornall, 1956) Rats fed a normal diet supplemented with 2 % desiccated thyroid for 10–30 days also showed a 50 % increase in liver G 6 Pase activity when the results were expressed as moles of P released per total liver wet weight and 20 % when expressed per total liver nitrogen content (Maley, 1957)

It is possible that the glucokinase glucose 6 phosphatase system could easily serve as a device for draining off high energy phosphate into useless work, since a single glucose molecule may be repeatedly phosphorylated and dephosphorylated at the expense of one adenosine triphosphate per trip An increase in G 6 Pase activity in thyrotoxicosis suggests that this system may be accelerated and thus contribute to an over all decreased cellular efficiency of energy utilization

6 *Summary of Hormonal Effects*

The biochemical behavior of hepatic G 6 Pase is in good agreement with the physiological and clinical data of hyperactivity and hypoactivity of the adrenal and pituitary glands It has long been recognized that adrenalectomized and hypophysectomized animals are unable to maintain their blood sugar level when subjected to fasting The demonstration of decreased liver G 6 Pase activity in fasted hypophysectomized or adrenalectomized animals (Weber and Cantero, 1957c) may offer an explanation for the falling

blood sugar The increased liver G 6 Pase activity after cortisone administration (Weber *et al*, 1956) may partially explain the increase in the rate of glucose production after cortisone treatment

Experimental studies of the diabetic syndrome have emphasized the existence of a hormone balance, involving the hypophysis, adrenal cortex, and pancreas, that participates in the regulation of carbohydrate metabolism in normal animals The results of the studies on liver G 6 Pase are consistent with the classical experiments, which clearly established the interdependence of the endocrine organs in regulating carbohydrate metabolism (Houssay, 1929, Long and Lukens, 1936) The agreement is well illustrated in experiments (Ashmore *et al*, 1956b) which showed that administration of adrenal cortical hormone caused a greater increase in liver G 6 Pase activity in the adrenalectomized diabetic animals than it did in normal rats On the other hand, injection of insulin to adrenalectomized animals resulted in a more marked decrease in G 6 Pase activity in these animals than it did in normal ones

The observation that hypophysectomy causes a more marked decrease in liver G 6 Pase activity than does adrenalectomy (Weber and Cantero, 1957c), indicates that the decreased hepatic G 6 Pase activity in hypophysectomized animals can not be attributed solely to decreased or absent adrenocortical activity Since thyroxine will also cause an increase in G 6 Pase activity it is possible that the effect of hypophysectomy on hepatic G 6 Pase activity is mediated through at least two endocrine organs, the adrenal cortex and the thyroid gland (Weber and Cantero 1957c)

Clinical studies have long demonstrated the influence of hyper or hypo thyroid conditions on carbohydrate tolerance (Maley and Lardy, 1956) and on coexisting diabetes mellitus in humans (Joslin, 1935) Soskin and associates demonstrated that the administration of thyroxine to hypophysectomized dogs maintained a normal blood sugar level through long periods of fasting (Soskin *et al*, 1939) The demonstration of the effect of thyroxine on liver G 6 Pase is in agreement with the previously shown effects of this hormone on carbohydrate metabolism

7 *Effect of Fasting*

Changes in hepatic G 6 Pase activity in fasting rats and mice have been studied rather extensively There is approximately a twofold increase in the activity of this enzyme per gram wet liver on fasting for 24 hours (Ashmore *et al*, 1954 Langdon and Weakley, 1955, Weber and Cantero, 1954) (Fig 3) Since the liver must serve as the major source of blood glucose during long periods of fast (Duncan, 1952), it is not at all surprising that the enzyme involved in the immediate release of glucose from this tissue should be increased

The increase in hepatic G 6 Pase activity induced by fasting provides an

excellent example of how changes in an enzyme activity can be interpreted As mentioned previously, the increase is very marked when expressed per gram of wet liver A similar increase in activity is also observed if activity is expressed per milligram nitrogen, but no significant increase is observed on fasting if the results are expressed per average cell or per total liver (Table VI) (Weber *et al*, 1956)

In fasting there is a marked reduction in liver mass, but without apparent reduction in the number of liver cells (Weber and Cantero, 1958a) Although the amount of enzyme per liver or per cell remains relatively constant, its concentration in protoplasm is evidently increased since activity per milligram nitrogen shows an increase It is also to be noted that G 6

TABLE VI

CHANGES IN HEPATIC GLUCOSE 6 PHOSPHATASE ACTIVITY OBSERVED WITH FASTING AND DIETARY CHANGE

Diet	Glucose 6 phosphatase activity[b]			
	Per gm liver	Per 100 gm body wt	Per gm protein	Per av cell
Fasting 24 hours	153	110	145	108
High protein diet	146	165	150	—
High fat diet	180	162	142	—
High fructose diet	185	210	150	—
High galactose diet	185	170	190	—

[a] For the composition of the various diets see Freedland and Harper (1957)

[b] *Glucose 6 phosphatase activity is expressed as a per cent of the activity found in animals fed a basal diet*

Pase has the same intracellular distribution in liver from fasting as in liver from fed rats In each case 45–50 % of the total homogenate activity can be recovered in the microsomal fraction (Weber *et al*, 1956)

The role of the adrenal cortex in maintenance of G 6 Pase activity during fasting is indicated by the marked reduction of phosphatase activity in livers from fasting adrenalectomized and fasting hypophysectomized rats (Froesch *et al*, 1958, Weber and Cantero, 1955c, 1957c)

Studies on enzyme changes during 1 day (Weber and Cantero, 1956a) or prolonged fast in rats (6 days) (Weber and Cantero, 1958a) showed that hepatic G 6 Pase markedly increased, whereas phosphoglucomutase and phosphohexoseisomerase apparently did not change when expressed on a wet weight basis However, when expressed per average cell, it was found that G 6 Pase activity was maintained at normal values during 6 days of fasting During the same period phosphohexoseisomerase and phosphoglucomutase progressively decreased, and about 70 % of their activity was lost

by the end of 6 days' fasting Essentially similar results were obtained when enzymatic activities were expressed per 100 gm body weight

8 Effect of Diet

Changes in G 6 Pase activity have been observed in rats fed on purified diets low in glucose content (Freedland and Harper, 1957) For example, substitution of isocaloric equivalents of protein fat, galactose, or fructose for the dietary glucose results in a marked increase in hepatic G 6 Pase activity calculated per gram of liver, per unit of body weight or per gram of liver protein These data are summarized in Table VI Fasting for 24 hours before the start of the experimental diet did not affect these results However no significant increase in hepatic G 6 Pase activity could be demonstrated in rats fed for 4 days on a protein free diet (Freedland and Harper 1958a)

Freedland and Harper (1958a) have found that inclusion of 31 caloric per cent of the diet as dextrin prevents increased G 6 Pase activity in response to both high protein and high fat diets The authors suggest that this amount of glucose, 31% of the daily caloric intake, may well represent the amount of glucose needed to maintain blood glucose and to satisfy other body requirements without unduly stimulating gluconeogenesis Freedland and Harper (1958a b) have further found that inclusion of as little as 6% fructose in the diet will cause an increase in hepatic G 6 Pase activity, whereas 50% galactose in the diet is required to produce a similar effect

Such changes in liver G 6 Pase activity in response to dietary changes have been described as metabolic adaptations (Freedland and Harper, 1958b) However, it is not as yet established whether these changes in enzymatic activity represent 'adaptation" to an increase in substrate or whether such changes with dietary intake are mediated by the endocrine glands For example, insulin release from the pancreas stimulated by feeding diets high in glucose could possibly result in lower hepatic G 6 Pase activity On the other hand, enzymatic changes observed in high fat and high protein diets may well be related to an increased elaboration of adrenal steroids rather than a ' primary adaptation" to the diet

The reverse of this argument has also been applied Harper (1956) reasons that since protein feeding results in increased G 6 Pase activity similar to that observed in the diabetic then it follows that in the diabetic the increased phosphatase activity probably represents an adaptation to a diminution in the availability of glucose rather than a direct response to a lack of insulin Such reasoning does not appear to stand up when applied to the decrease in activity observed in the adrenalectomized diabetic animal

or to the increased activity observed in animals treated with adrenal steroids

The effect of carcinogenic and control basal diet on liver G 6 Pase activity (Spain, 1956, Weber and Cantero 1955a) is discussed in Section VI

VI Pathology of Glucose 6 phosphatase

1 *In Glycogen Storage Disease*

Von Gierke's, or hepatic glycogen storage, disease, is thought to represent a congenital hepatic deficiency of glucose 6 phosphatase (Cori and Cori, 1952, Harris, 1952, Harris and Olmo, 1954, Schwartz *et al*, 1957) Absence of glucose 6 phosphatase activity in liver from von Gierke's disease obtained at autopsy (Cori and Cori, 1952), represents the first instance in which the activity of this particular enzyme was associated with a pathological state In order to rule out the possibility that G 6 Pase activity was decreased due to the presence of an inhibitor, homogenates of liver from glycogen storage patients were incubated with homogenates of normal liver (Cori and Cori, 1952) No inhibition could be detected over the pH range 5 to 9 That decreased activity might be due to absence of co factors was also ruled out by the addition of boiled extracts of normal liver to homogenates of liver from von Gierke's The almost complete absence of G 6 Pase activity from liver in glycogen storage disease was regarded as the possible primary cause of the clinical symptoms (Cori and Cori, 1952)

Cori and Cori have drawn attention to the fact that abnormal storage of glycogen in skeletal muscle or heart muscle cannot be explained by a lack of G 6 Pase since this enzyme is normally absent from these tissues Their suggestion that the activity of amylo 1,6 glucosidase be measured in these tissues has been followed up, and a deficiency of this enzymatic activity in muscle glycogen storage disease has been established (Illingworth *et al*, 1956)

Schwartz *et al* (1957) have reported clinical biochemical studies on a 3 month old infant who was diagnosed as a case of hepatorenal glycogen storage disease Liver biopsy was obtained at an exploratory laparotomy The liver homogenate was assayed for G 6 Pase activity by the method of Cori and Cori (1952), and no inorganic phosphate production from G 6 P was shown Addition of deoxycholic acid (10^{-3} *M*) to the incubation mixture resulted in no activation of the enzyme (Ashmore and Nesbett, 1955) The liver homogenate was also incubated with C^{14} glucose Glycogen breakdown during incubation with the formation of lactate was observed The specific activity of radioglucose was unchanged during the incubation, suggesting no dilution by glucose formation from glycogen or other pre

cursors The absence of G 6 Pase was also demonstrated by utilizing the intravenous galactose tolerance test The *in vivo* demonstration of impaired G 6 Pase activity was indicated by normal galactose utilization with increased lactate production and without an increase in blood glucose despite the presence of hypoglycemia

2 Attempts to Influence Hepatic Glucose 6 phosphatase in Glycogen Storage Disease

The use of thyroxine and adrenal cortical steroids in the treatment of von Gierke s disease has been suggested (Koulischer and Pickering 1956) However, there is no evidence as yet available to indicate that hormonal treatment will increase the activity of liver G 6 Pase in such cases Harris and Olmo (1954) examined hepatic G 6 Pase activity under various clinical conditions They observed that not only glycogen storage disease, but also other hepatic storage diseases affected G 6 Pase activity in this organ Cases of edema of the liver, passive congestion, fatty liver, biliary and Laennec's cirrhosis, and acute and chronic atrophy were accompanied by decreases in G 6 Pase activity commensurate with the degree of damage to the liver as observed histologically It was also noted that invasion of the liver by tumor tissue also caused a significant reduction in G 6 Pase activity in the liver tissue

3 In Neoplasia

Recently the behavior of G 6 Pase has been studied in homogenates of various tumor tissues (Weber and Cantero 1955a) No G 6 Pase activity was found in sarcoma 37 adenocarcinoma E 0771, or lymphosarcoma 6C3HED It is of special interest that G 6 Pase activity is absent in the Novikoff tumor The histological picture of the Novikoff tumor (Weber and Cantero 1955a) shows that one is justified in calling this tumor a hepatoma If this tumor were a cholangioma the increased number of bile duct cells which do not contain G 6 Pase might explain its lack of G 6 Pase activity However, since this tumor is composed of parenchymal cells, which normally contain much G 6 Pase (Chiquoine, 1953), the absence of activity is real and not caused by a change in the cell population of the tissue (Weber and Cantero, 1955a)

These studies of neoplastic tissues were done on homogenates prepared in isotonic sucrose Investigation was also carried out to determine whether G 6 Pase activity could be demonstrated under different assay conditions Since most of the normal liver G 6 Pase activity is concentrated in the microsomal fraction the possibility arose that in a homogenate prepared in isotonic sucrose the hepatoma microsomes were not broken and G 6 Pase could not be liberated or the substrate could not reach the enzyme

for 150 days, followed by a period of 30 days on Purina Fox Chow The enzymatic activity was expressed on wet weight and nitrogen basis and also in per cent change as compared with the normal, fox chow fed animals The feeding of the control semisynthetic diet increased liver G 6 Pase activity On the other hand, the G 6 Pase activity of the liver progressively decreased during DAB feeding, and at 180 days the activity was almost completely absent from the induced hepatomas The difference between

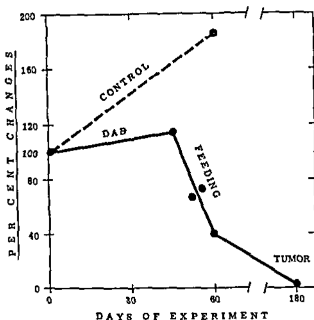


FIG 4 Glucose 6 phosphatase activity during carcinogenesis Rats were on DAB diet for 150 days then on fox chow for 30 days Activity is expressed as per cent change from control fox chow fed rats killed at the same time Each point represents the average of at least three animals Reprinted from Weber and Cantero (1955a)

the values of control and DAB fed rats was significant on both wet weight and nitrogen basis (see Fig 4)

Since the semisynthetic diet (Allard *et al*, 1953) has a very high carbohydrate content the increased G 6 Pase activity might be considered an adaptive response of the organism (Weber and Cantero, 1955a) On the other hand when the carcinogenic dye was incorporated into the semisynthetic diet very little increase was observed and after 40 days the enzyme activity progressively decreased The almost complete absence of G 6 Pase activity in the induced hepatoma agrees well with the finding that this enzyme is absent in the transplanted Novikoff hepatoma

The decrease in hepatic G 6 Pase activity during azo dye carcinogenesis was recently confirmed by Spain (1956) In these studies 3′ methyl-4

However, Novikoff hepatoma homogenates prepared in isotonic NaCl or in distilled water also had no G 6 Pase activity Preparation of more concentrated (20%) or highly diluted (1%) hepatoma homogenate also failed to show any G 6 Pase activity (Weber and Cantero, 1955a, b)

It was also shown that the absence of G 6 Pase activity in neoplastic tissues is not due to the presence of an inhibitor, since homogenates of adenocarcinoma or hepatoma did not inhibit reaction mixtures containing normal liver A 1 hour preincubation of various tumor homogenates with normal liver homogenate, prior to the enzyme assay, was also without any effect on the G 6 Pase activity of the normal liver (Weber and Cantero, 1955a)

4 Attempts to Induce or Restore Glucose 6 phosphatase Activity in Neoplastic Liver

Since fasting greatly increases normal liver G 6 Pase activity (Ashmore *et al*, 1954, Langdon and Weakley, 1955, Weber and Cantero, 1954, Weber *et al*, 1955, 1956), rats bearing 6 day old transplanted Novikoff hepatomas were fasted for 24 hours and then sacrificed The livers of these rats showed a 60% increase in G 6 Pase activity per wet weight, but again no activity could be demonstrated in the intraperitoneally transplanted hepatoma (Weber and Cantero, 1957b)

Cortisone administration has been shown to result in an increased liver G 6 Pase activity (Ashmore *et al*, 1957b, Langdon and Weakley, 1955, Weber *et al*, 1955, 1956), however, injections of high doses of cortisone (25 mg per rat of 100 gm weight) for 5 days failed to cause any demonstrable G 6 Pase activity in the Novikoff hepatoma (Weber and Cantero, 1957b)

The results of these studies on the effect of fasting and cortisone administration on G 6 Pase activity of Novikoff hepatoma showed that either the enzyme was completely absent in this tumor or that the hepatoma cells did not react to these physiological stimuli in the same way as normal liver cells did (Weber and Cantero, 1957b)

5 In Carcinogenesis

The behavior of G 6 Pase activity has also been examined during carcinogenesis and in the resulting primary tumors (Weber and Cantero, 1955a) Precancerous livers were taken from male Wistar rats of 200 gm, which were fed up to 150 days a semisynthetic diet in which 4 dimethyl aminoazobenzene (DAB) was incorporated at a concentration of 0 06% Control animals received the semisynthetic diet alone Rats were sacrificed at different intervals after they had been on the diets for 50–60 days Primary induced liver tumors were obtained from rats fed the DAB diet

for 150 days, followed by a period of 30 days on Purina Fox Chow The enzymatic activity was expressed on wet weight and nitrogen basis and also in per cent change as compared with the normal, fox chow fed animals *The feeding of the control semisynthetic diet increased liver G 6 Pase activity* On the other hand, the G 6 Pase activity of the liver progressively decreased during DAB feeding, and at 180 days the activity was almost completely absent from the induced hepatomas The difference between

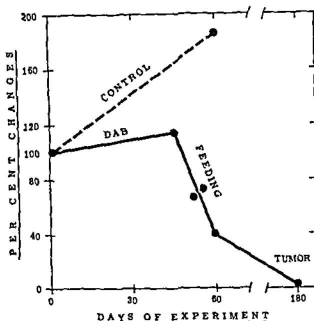


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The decrease in hepatic G 6 Pase activity during azo dye carcinogenesis was recently confirmed by Spain (1956) In these studies 3′ methyl-4

dehydrogenase (Weber *et al*, 1956) runs parallel with the regeneration of G 6 Pase (Weber and Cantero, 1957b) These three enzymes are localized in the nonparticulate fraction (Glock and McLean, 1953, Hers *et al*, 1951)

TABLE VIII*

AMOUNT OF G 6 P METABOLIZED *in Vitro* UNDER OPTIMAL PH AND SUBSTRATE CONDITIONS BY HUMAN AND RAT LIVER ENZYMES

Enzymes	Activity per gm wet weight (μmoles/hour)		Activity per mg nitrogen (μmoles/hour)	
	Rat	Human	Rat	Human
Phosphohexoseisomerase	10730 ± 1733 (100)	15630 ± 5241 (100)	715 ± 95 (100)	1122 ± 376 (100)
Phosphoglucomutase	6932 ± 803 (65)	10149 ± 5046 (65)	463 ± 51 (65)	711 ± 312 (64)
Glucose 6 phosphatase	463 ± 49 (4 3)	255 ± 118 (1 6)	14 4 ± 1 5 (2 0)	8 4 ± 3 8 (0 8)
G 6 P dehydrogenase	65 ± 24 (0 6)	90 ± 24 (0 6)	2 3 ± 0 9 (0 3)	5 2 ± 1 4 (0 7)
Ratios				
Phosphohexoseisomerase / phosphoglucomutase	1 55	1 54	1 55	1 55
Glucose 6 phosphatase / glucose 6 phosphate dehydrogenase	7 1	2 8	6 3	1 6

From Weber and Cantero (195 e)

[a] The means and standard deviations of six or more specimens are given. Results in parentheses are expressed in per cent the values of phosphohexoseisomerase are taken arbitrarily as 100%

9 Hepatic Glucose 6-phosphatase in Adult Human Liver

Comparison of human and rat liver values The activity of G 6 Pase, phosphoglucomutase, phosphohexoseisomerase, and glucose 6 phosphate dehydrogenase in normal human liver has been measured (Weber and Cantero, 1957e) The results of the study on these four hepatic enzymes have been compared with those of the rat liver enzymes (Table VIII)

The *human liver* specimens were obtained by biopsy at abdominal operations from patients with clinical diagnosis of cholecystitis and cholelithiasis Hematoxylin and eosin slides were made of each specimen Only those biopsy results which showed normal liver structure on histological examination were included in the series *Rat livers* were obtained from male adult

animals of 200 gm weight G 6 Pase was assayed in the homogenate, phosphoglucomutase, phosphohexoseisomerase, and glucose 6 phosphate dehydrogenase (Kelley *et al*, 1955) were assayed in the supernatant fluid The enzymatic activities were expressed per wet weight and nitrogen Proportionality to time and amount of enzyme at optimal substrate concentration was established for all four enzymes In Table VIII the enzymes involved in G 6 P utilization are compared at optimal pH, with substrate present in excess and at 37° C Under these circumstances in the *rat*, liver phosphohexoseisomerase and phosphoglucomutase are the most powerful enzymes while G 6 Pase activity is comparatively quite low and that of G 6 P dehydrogenase is the lowest

It is interesting to note that the G 6 P utilizing enzymes of human liver show a remarkable parallelism with the rat liver enzymes The absolute values of G 6 Pase are lower in human liver On the other hand human hepatic phosphohexoseisomerase, phosphoglucomutase and G 6 P dehydrogenase activities are higher than the corresponding rat liver enzymes The phosphohexo eisomerase phosphoglucomutase ratio was 1 55 in both rat and human liver on nitrogen basis However, the G 6 Pase G 6 P dehydrogenase ratio was 6 3 in the rat and only 1 6 in human liver It is of importance to note that the G 6 Pase G 6 P dehydrogenase ratio of rat liver (on wet weight basis) (7 1) agrees well with the ratio (6 7), which may be calculated from the data of Perske *et al* (1957) in normal rat liver

Increased G 6 Pase activity has been noted in liver biopsy samples from thirteen human diabetics (Patrick and Tulloch, 1957) Stabilization of the diabetes was followed by decreased activity of the enzyme, whether insulin or carbutamide had been used to control the diabetes

VII Miscellaneous

1 Effect of Sulfonylureas on Glucose-6 phosphatase

Attention has recently been focused on a new series of drugs termed sulfonylureas that have the ability to produce hypoglycemia when administered to normal and to some diabetic animals One of the proposed actions for these drugs is that they inhibit hepatic glucose production The mechanism of action of the sulfonylureas is not within the scope of this review, however, there is considerable evidence from several laboratories indicating that chronic administration of these drugs to rats will produce a significant decrease in hepatic glucose 6 phosphatase activity (Ashmore *et al*, 1956a Hawkins *et al* 1956, Haist *et al* 1957 Kuether *et al*, 1957, Tyberghein *et al* 1956) The administration of tolbutamide (1 butyl 3 tolylsulfonyurea) to normal rats over a period of 48 hours results in a decrease in liver glucose 6 phosphatase activity to 60–70% of normal (Ashmore *et al*, 1956a)

There is some question as to whether or not an inhibition of glucose 6 phosphatase activity can account for the hypoglycemia observed, since administration of a single dose of tolbutamide to rats produces a pronounced hypoglycemia within 3 hours, yet during this time *no diminution* in glucose 6 phosphatase activity was observed (Ashmore *et al*, 1956a, Kuether *et al*, 1957)

When added to liver preparations *in vitro*, sulfonylureas have been shown to decrease glucose production, decrease the rate of glycogenolysis (Berthet *et al*, 1956, Vaughn, 1956), and inhibit glucose 6 phosphatase (Ashmore *et al*, 1956a, Haist *et al*, 1957, Tyberghein *et al*, 1956) However, the drug concentration required to produce these effects *in vitro* is in excess of the concentrations required to produce *in vivo* effects It is of interest that although sulfonylureas inhibit liver G 6 Pase and phosphohexoseisomerase activity *in vitro* only in high concentrations (0 5–5 0 mg per reaction mixture), the other enzymes (phosphoglucomutase and G 6 P dehydrogenase) which are involved in G 6 Pase utilization are not affected by the drug under the same conditions (Weber and Cantero, 1958b) This subject has recently been reviewed in more detail (Ashmore *et al*, 1957a)

2 Glucose 6-phosphatase in Chick Liver Microsomes

Le Clerc (1954) has used G 6 Pase activity to estimate the microsomal content of liver preparations In order to test the possibility that microsomes multiply like viruses, the microsomes of chick liver were inoculated on chorioallantoic membranes G 6 Pase activity was determined in the treated membranes, and it was found that this enzymatic activity decreased during the first 24 hours after inoculation However, after 48 hours G 6 Pase activity increased to two to six times the value attained after 24 hours A suspension of heated liver granules or of heated *Tetrahymena geleii*, though producing the same lesions of the membrane did not increase the G 6 Pase activity of the microsome fraction after 48 hours The author concluded that these results were compatible with the idea that liver microsomes multiplied by autoduplication

3 Effect of Shock on Liver Glucose 6 phosphatase

Moyson and Gavosto described an increased G 6 Pase activity after shock in the microsomal fraction of rat liver This increased G 6 Pase activity paralleled the hyperglycemia in the shocked animals (Moyson and Gavosto, 1953)

4 Glucose 6-phosphatase Study in Tissue Culture

Perske *et al* (1957) have reported studies on carbohydrate enzymes of culture strains of human liver Homogenates prepared from these cultures showed no G 6 Pase activity Other hepatic enzymes not present in tissue

culture cells were fructose diphosphatase fructokinase, and glucokinase On the other hand, G 6 P dehydrogenase activity was present in these cells

5 *Glucose-6 phosphatase in Scurvy*

Lahiri and Banerjee studied the effect of ascorbic acid deficiency on G 6 Pase in guinea pig (Lahiri and Banerjee, 1956) Female guinea pigs (230–260 gm) were fed a scorbutic diet, with or without supplement of ascorbic acid, by paired feeding Pairs of animals were sacrificed after an overnight fast on twenty fifth day of experiment G 6 Pase was assayed according to Swanson (1950), and activity was expressed on a dry weight basis

It was shown that G 6 Pase activity of the hepatic tissue was significantly increased in scurvy At the same time the G 6 Pase content of the liver was significantly decreased Kidney G 6 Pase was unaffected

6 *Effect of X Irradiation on Glucose 6 phosphatase*

The effect of 600 r total body X irradiation was studied in normal and hypophysectomized rats It was found that hepatic G 6 Pase activity was not affected at 1, 3, 7, and 8 days after irradiation (Weber and Cantero, 1957f, Coniglio *et al*, 1957) Under *in vitro* conditions, X irradiation in doses up to 20 000 r failed to effect G 6 Pase activity of homogenates prepared in isotonic saline, sucrose, or distilled water (Weber and Cantero, 1959a b)

VIII Summary and Conclusions

Hepatic glucose 6 phosphatase activity can be altered by a number of dietary and endocrine manipulations The activity of this enzyme is always increased under conditions giving rise to an increased hepatic glucose production, or gluconeogenesis However, in several instances an increased hepatic glucose production precedes by several hours any measurable change in the activity of the hepatic phosphatase In any consideration of enzymatic activity as a factor in the regulation of metabolic activity, it should be borne in mind that enzymatic activity is usually measured under condition where the substrate is present in great excess Such conditions most likely do not obtain within the cell It is, therefore essential to know the substrate concentration as well as the total enzymatic activity and affinity of enzyme for the substrate before any complete evaluation of the role of an enzyme in metabolic regulation can be made At present these data are not all available

Studies to date on the endocrine regulation of G 6 Pase activity have raised many interesting questions Do such changes in activity represent "metabolic adaptation" or do they represent hormonal regulation of the synthesis of a specific protein? Here again the investigator meets with in

complete knowledge of the system under study However such problems can be resolved and with continued experimental attack will yield answers that may prove to be of fundamental importance in our understanding of the mechanism of hormone action and the over all problem of metabolic regulation Because of the wealth of data already available and of the ease with which activity can be associated with function, we feel that G 6 Pase represents the ideal focal point for such an experimental attack

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Biochemistry of Serotonin and Other Indoleamines

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Page

I Introduction

When Wieland *et al* (1934) and Jensen and Chen (1936) isolated bufotenine (*N*,*N* dimethyl 5 hydroxytryptamine) and related indoleamines from the toad venom gland, they considered them as poisons peculiar to this species of animal About twenty years elapsed before Rapport *et al* (1948) isolated serotonin (5 hydroxytryptamine) from beef blood and determined its structure (Rapport, 1949) With the isolation of serotonin the full significance of the indoleamines in nature began to unfold

Although the exact functions of serotonin are not known, it has been implicated in a variety of physiologic and pathologic conditions including gastrointestinal activity, the function of the central nervous system anaphylaxis, kidney function, etc These aspects of serotonin have been excellently covered by Page (1958) in his recent review

Tryptamine formation was first reported in animal tissues by Werle and Mennicken in 1937 Although it was reported in urine of pellagrins by Sullivan (1922), its presence in normal human urine (Rodnight 1956, Sjoerdsma *et al*, 1959) and in animal tissues (Hess *et al*, in press) was only recently shown Its physiologic significance also remains to be deter

mined Indoleamines hydroxylated in other than the 5 position have been reported to be formed enzymatically (Ichihara *et al*, 1957) from tryptamine The identity of these products and their physiological significance remain to be determined

This report will attempt to present the available evidence for the chemical transformations starting from tryptophan and to discuss the properties of the various tissue catalysts responsible for these chemical changes It will also discuss those biochemical findings which have specific physiologic, pharmacologic, and clinical implications

A biochemical report must usually present the methodology, both qualitative and quantitative, used in the studies However, the analytical pro

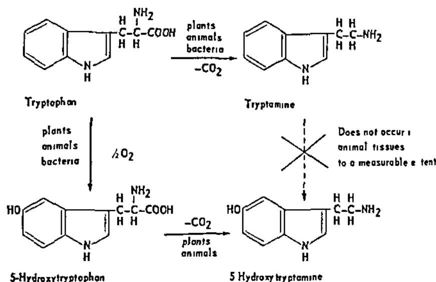


FIG 1 Pathway of serotonin formation

cedures for serotonin and its metabolites have been recently summarized (*Udenfriend et al*, 1958) Chemical methods for tryptamine (Sjoerdsma *et al* 1959) and its metabolite, indoleacetic acid (Weissbach *et al*, 1959), have also been presented An excellent review of paper chromatography applied to indole compounds is presented in "Chromatographic Techniques" by J B Jepson (1958)

II BIOGENESIS OF SEROTONIN

A summary of our present knowledge of serotonin formation is presented in Fig 1

That tryptophan serves as the dietary precursor of serotonin has been adequately demonstrated both by radioactive studies in animals (*Udenfriend et al*, 1956) and by balance studies in patients with malignant carcinoid (Sjoerdsma *et al*, 1956) In the latter case as much as 60% of the

daily tryptophan intake was shown to be excreted as 5 hydroxyindoleacetic acid (5HIAA),[1] the major end product of serotonin metabolism Recently it has been shown that cells obtained from culturing mouse mast cells in tissue culture can readily convert tryptophan to serotonin (Schindler, 1959)

The conversion of tryptophan to serotonin requires both decarboxylation and hydroxylation of the aromatic ring Two routes are, therefore, conceivable (1) Hydroxylation to yield 5 hydroxytryptophan (5HTP), followed by decarboxylation or, (2) decarboxylation to yield tryptamine, followed by hydroxylation at the 5 position

1 Evidence for 5 Hydroxytryptophan Pathway

The evidence for this pathway is now fairly well established First both tryptophan and 5HTP give rise to serotonin in animal tissues (Udenfriend *et al*, 1956) 5HTP decarboxylase, which is found in almost all organ tissues, is a highly active enzyme The amino acid has been found in bacteria (Mitoma *et al* 1956) and in toads (Udenfriend *et al*, 1956) where its conversion from tryptophan was also demonstrated The urine of certain patients with malignant carcinoid[2] has been found to contain relatively large amounts of 5HTP (Dalgliesh 1956 Sandler and Snow 1958) Presumably such tumors secrete the amino acid precursor of serotonin However, tumors of this type have not as yet been obtained for biochemical study to confirm the urinary findings

2 Tryptamine Pathway

The finding of tryptamine in tissues suggests the possibility of its conversion to serotonin However, the following experiments (Udenfriend *et al*, 1959) indicate that it does not serve as a precursor of serotonin

When large amounts of tryptamine were administered to animals, the serotonin contents of tissues were not increased Administration of potent inhibitors of monoamine oxidase (MAO) along with tryptamine to prevent its destruction was also without effect Tryptamine administration did not result in an increased excretion of the serotonin metabolite, 5HIAA On the other hand administration of comparable amounts of either tryptophan or 5HTP does increase tissue levels of serotonin and urinary excretion of 5HIAA

When C^{14} labeled tryptamine was administered to rabbits, the serotonin isolated from platelets stomach and intestine was found to be devoid of

[1] The following abbreviations are used 5HTP 5 hydroxytryptophan 5HIAA 5 hydroxyindoleacetic acid MAO monoamine oxidase DPN diphosphopyridine nucleotide ATP adenosine tripho phate Dopa 3 4 dihydroxyphenylalanine

[2] Malignant carcinoid is a tumor derived from enterochromaffin cells the tumor like the parent cells synthesizes and secretes serotonin

mined Indoleamines hydroxylated in other than the 5 position have bee reported to be formed enzymatically (Ichihara *et al*, 1957) from tryptamine The identity of these products and their physiological significance remai to be determined

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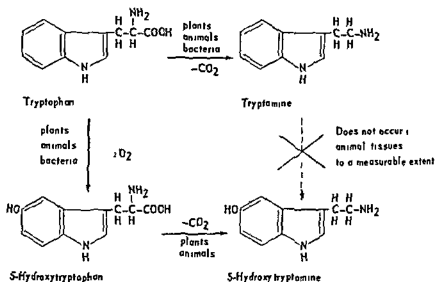


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The conversion of tryptophan to serotonin requires both decarboxylation and hydroxylation of the aromatic ring Two routes are therefore, conceivable (1) Hydroxylation to yield 5 hydroxytryptophan (5HTP), followed by decarboxylation, or, (2) decarboxylation to yield tryptamine, followed by hydroxylation at the 5 position

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When C^{14} labeled tryptamine was administered to rabbits the serotonin isolated from platelets stomach, and intestine was found to be devoid of

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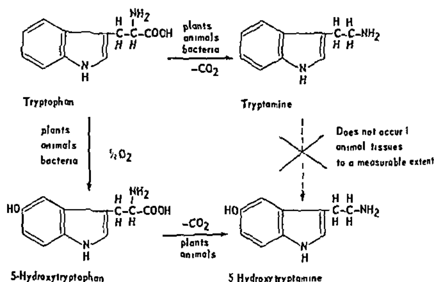


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radioactivity In contrast to this, serotonin isolated following administration of C^{14} labeled tryptophan or 5HTP has been found to be highly labeled

There remains the report of Ichihara *et al* (1957) claiming that the microsome rich supernatant fraction of liver homogenates hydroxylated tryptamine to yield both 5 and 7 hydroxytryptamine In the reviewer's laboratory attempts to demonstrate 5 hydroxylation of tryptamine by microsomes have been without success (Udenfriend *et al*, 1959) With specific and sensitive colorimetric and fluorometric procedures no trace of serotonin could be found following incubation of microsomes with tryptamine and TPN Other attempts to corroborate the conversion of tryptamine to serotonin by microsomes have also been without success[3] Apparently microsomes do hydroxylate tryptamine since both of the above groups have corroborated the finding that phenolic indole material is formed, as reported by Ichihara *et al* (1957) However, the identity of the phenolic compound or compounds remains to be determined

III Formation of 5-Hydroxytryptophan

The oxidation of tryptophan to 5HTP is analogous to the conversion of phenylalanine to tyrosine All the known mammalian aromatic hydroxylating enzymes have thus far been found only in liver These include phenylalanine hydroxylase, kynurenine hydroxylase, and the nonspecific microsomal aromatic hydroxylase None of these can convert tryptophan to 5HTP It has thus far not been possible to demonstrate enzymatic conversion of tryptophan to 5HTP by mammalian tissues *in vitro* Dalgliesh (1957), using elegant whole organ perfusion experiments, was not able to detect conversion of C^{14} tryptophan to 5HTP or serotonin by rat liver or intestinal mucosa

Although conversion of tryptophan to 5HTP is not readily detectable in animal tissues, there are microorganisms which carry out this oxidation as a major route of tryptophan metabolism This is the case in *Chromobacterium violaceum* (Mitoma *et al*, 1956), where it appears that a large proportion of the tryptophan is converted first to 5HTP and then to other products which may ultimately lead to the purple pigment, violacein (De Moss and Evans, 1958) The hydroxylase in this organism is specific for tryptophan and forms only the *l* form of 5HTP No phenolic products were observed which would indicate hydroxylation in positions other than the 5 position Many studies have been carried out with *C violaceum* using intact cells However, attempts to solubilize the enzyme have thus far met with failure, so that there is as yet little information concerning the mech

[3] Dr Stephen Szara, National Institute of Mental Health St Elizabeth s Hospital, Washington, D C

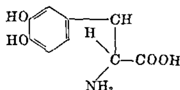
anism of the bacterial hydroxylation The isolation, purification, and characterization of tryptophan hydroxylase remains as the most important unfinished aspect of the 5 hydroxyindole route of tryptophan metabolism

Since the formation of 5 hydroxyindoles is markedly increased in patients and animals with nonhepatic tumors it would appear that tryptophan hydroxylase must occur in the tumor Thus it may be that, unlike other aromatic hydroxylases, tryptophan hydroxylase is present in all tissues where serotonin is formed In this respect it may resemble tyrosine hydroxylase, which converts tyrosine to 3 4 dihydroxyphenylalanine This reaction occurs in adrenal medulla (Rosenfeld *et al*, 1958) and in other tissues which form noradrenaline, such as sympathetic nerves and ganglia (Goodall and Kirshner, 1958)

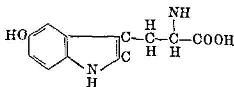
IV Conversion of 5 Hydroxytryptophan to Serotonin

The first evidence for 5HTP as an intermediate in serotonin formation was the demonstration of potent 5HTP decarboxylase activity in tissues This enzyme was purified from guinea pig and hog kidney and shown to be separable from 3 4 dihydroxyphenylalanine(dopa) decarboxylase By procedures involving differential heat inactivation salt fractionation, and adsorption on alumina it was possible to change the ratio of dopa decarboxylase to 5HTP decarboxylase by as much as twentyfold (Clark *et al*, 1954)

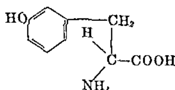
It is important to keep these facts in mind since both decarboxylases are widely distributed in animal tissues Not only that, but each has been found in tissues in which it supposedly has no function Thus 5HTP decarboxylase has been found in adrenal medulla and pheochromocytoma tumors which produce only epinephrine and norepinephrine, and dopa decarboxylase has been found in carcinoid tumors which produce only serotonin Furthermore structural analogs of dopa which have been found to inhibit dopa decarboxylase also inhibit 5HTP decarboxylase (Holtz, in press, Clark, 1959) Not only that, but in homogenates each compound inhibits the decarboxylation of the other (Holtz in press, Clark, 1959) Structurally dopa and 5HTP are quite similar (formulas I and II) Each can be regarded as related to metatyrosine (III), which is also readily decarboxylated by mammalian tissues It may be this similarity between the two amino acids which results in their interaction with the two enzymes However, although dopa and 5HTP decarboxylase activities are seldom found separately in mammalian tissues the ratio of one to the other varies from tissue to tissue, and this ratio can be further varied by standard enzyme purification procedures It seems fairly certain that 5HTP decarboxylase and dopa decarboxylase are two distinct enzymes It is also of interest that both dopa and *m* tyrosine are decarboxylated by *Streptococcus faecalis* extracts whereas



3,4 Dihydroxyphenylalanine
(I)



5 Hydroxytryptophan
(II)



m Tyrosine
(III)

5HTP is not (Clark *et al*, 1954) Other factors which argue for two separate enzymes are (1) tremendously different pH optima, 6 9 for dopa and 8 1 for 5HTP, (2) tremendously different substrate enzyme affinities, and (3) marked differences in their abilities to dissociate from the coenzyme, pyridoxal phosphate

V Properties of 5 Hydroxytryptophan Decarboxylase

5HTP decarboxylase is a soluble enzyme which is precipitated by 55% ammonium sulfate The affinity of substrate is so high that the enzyme exhibits zero order kinetics at the lowest substrate concentrations which permit measurement, 10^{-5} *M* Pyridoxal phosphate is also bound fairly firmly to the enzyme, and in initial studies with guinea pig enzyme little resolution was possible Buzard and Nytch (1957) showed a direct pyridoxal phosphate requirement of this enzyme Subsequently, using rat tissues, fairly complete separation of coenzyme from the decarboxylase was demonstrated by Buxton and Sinclair (1956) and by Weissbach *et al* (1957a) Following this it was shown that enzyme activity could be completely restored by adding pyridoxal phosphate to the resolved enzyme

In addition to pyridoxal phosphate it has been reported that 5HTP decarboxylase also seems to require a metal This metal requirement stems from the finding that chelating agents such as ethylenediamine tetraacetate inhibit activity (Beiler and Martin, 1954) Inhibition by chelating agents has been confirmed in the reviewer s laboratory

Inhibition of 5HTP decarboxylase has been suggested as the mechanism of action of one hydrazinophthalazine (Perry *et al*, 1955, von Schuler and Meier, 1955), a potent hypotensive agent However, no supporting evidence is given for this action in the article Studies in the author s laboratory have corroborated this report and indicate that 1 hydrazinophthalazine can

indeed inhibit 5HTP decarboxylase *in vivo* The significance of this action with respect to the hypotensive action of the drug remains to be determined Davison and Sandler (1958) have suggested that inhibition of 5HTP decarboxylase may also explain the somewhat lower levels of serotonin found in patients with phenylketonuria (Pare *et al* 1957) It was shown (Davison and Sandler, 1958) that phenylalanine metabolites such as phenylpyruvic, phenyllactic, and phenylacetic acids can inhibit 5HTP decarboxylase The suggestion is made that since these acids are produced in large amounts in phenylketonuria they may be responsible for the mental defect by blocking 5HTP decarboxylase However the concentrations used to achieve detectable inhibition are greater than 0.02 *M* It is doubtful whether such concentrations of any metabolite is ever reached in the tissues

VI In Vivo Findings Related to 5 Hydroxytryptophan Decarboxylase

5HTP decarboxylase has been found in all tissues which contain serotonin In the central nervous system the localization of 5HTP decarboxylase parallels the localization of serotonin (Gaddum and Giarman, 1956, Bogdanski *et al* 1957) Tissues such as cerebellum, which contain little serotonin contain but traces of 5HTP decarboxylase, whereas hypothalamus is rich in both From these and other considerations it would appear that serotonin is made centrally

TABLE I
SEROTONIN LEVELS IN NORMAL AND VITAMIN B_6 DEFICIENT CHICKS [b]

Tissue	Serotonin	
	Control (μg /gm)	Vitamin B_6 deficient (μg /gm)
Brain	1.1 (±0.3)	0.4 (±0.06)
Intestine	5.3 (±1.2)	1.2 (±0.4)
Liver	1.6	0.1
Blood	2.9	0.5 (±0.08)

Taken from Weissbach *et al* (195)
[b] The chicks used in this experiment were those made chronically deficient and their pair fed controls Unless otherwise stated each value represents the average obtained from six animals The values in parentheses represent the mean deviation
Tissues from four animals were pooled and analyzed

It has also been possible to show marked diminution in the serotonin depots of chicks which were raised from hatching on a vitamin B_6 deficient diet (Weissbach *et al*, 1957a) As shown in Table I the amount of serotonin in all tissues was markedly diminished When 5HTP was adminis

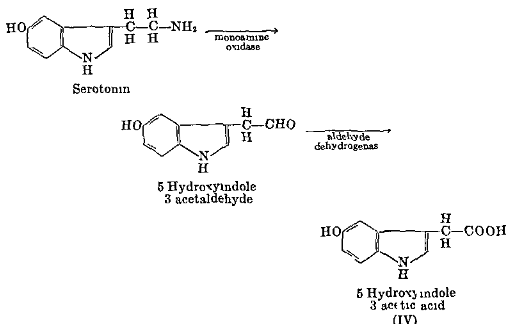
tered to the B_6 deficient chicks they also formed less serotonin than did the controls

The most interesting findings with respect to 5HTP concern its ability to penetrate into tissues, including the brain, probably by the common mechanisms of amino acid transport As a result, it is converted by the decarboxylase in the tissues to serotonin (Udenfriend *et al*, 1957) In this manner it has been possible to raise the levels of tissue serotonin manyfold Since brain levels are simultaneously increased, marked central effects are produced which have been likened to those observed by stimulation of the mesencephalon, diencephalon, and rhinencephalon (Bogdanski *et al*, 1958), areas which regulate behavioral and autonomic functions Administration of 5HTP also results in elevation of serotonin in many other organs 5HTP has been administered to rats and to patients as a means of elevating tissue serotonin levels for pharmacological and clinical studies (Davidson *et al*, 1957) In this respect, its administration is much superior to the administration of serotonin itself since in the former case the amine is found more widely distributed, including the central nervous system, and tissue levels persist for longer periods of time Woolley (1958) has made use of the serotonin formed through 5HTP administration and decarboxylase action as a sensitive method for testing antiserotonin activity *in vivo*

VII Metabolism of Serotonin

Many pathways for serotonin metabolism have been demonstrated and even more have been postulated However, in man and in most mammals oxidative deamination appears to be the major route of serotonin destruction Of an orally administered dose of serotonin in man 85–95 % is excreted in the urine as 5HIAA within several hours (Sjoerdsma *et al*, 1958) Following intravenous administration in man, as much as 65–80 % can be excreted as the acid metabolite (Davidson *et al*, 1957) However, the percentage of an administered dose excreted as 5HIAA has also been reported to be much less (30–40 %) by others (Erspamer, 1954a, Ferrari and Castelli, 1954) It may well be that the route of administration is a deciding factor In most laboratory animals the conversion of serotonin to 5HIAA is also extremely low These findings have been taken to indicate that in many patients and in certain animal species other routes of metabolism may be more important than oxidative deamination However, as shown below, the conversion of serotonin to 5HIAA (formula IV) is a two step process, and it may be that under some conditions and in some species the second step in the reaction may be limiting, so that the intermediate aldehyde is converted to products other than 5HIAA

Nakai (1958) has demonstrated that in herbivores the low excretion of 5HIAA can be explained by pigment formation from the intermediate



Serotonin

5 Hydroxyindole 3 acetaldehyde

5 Hydroxyindole 3 acetic acid (IV)

aldehyde Such pigment formation was previously reported by Blaschko and Hellmann (1953) Nakai (1958) goes on to state that the inability of "whole" herbivores to produce 5HIAA suggests that the bulk of diphosphopyridine nucleotide (DPN) *in vivo* may not be available for aldehyde oxidation A similar situation may exist in other animal species including man

The enzyme referred to as MAO was first described by Hare in 1928 A detailed review of studies on MAO and their physiological implications has recently appeared (Davison, 1958) Since serotonin is a primary aliphatic amine it was not surprising to find that it was an excellent substrate for this enzyme (Sjoerdsma *et al*, 1955 Zeller *et al*, 1955) The most active preparations of MAO are mitochondria from liver and kidney When serotonin is incubated with such preparations it is rapidly destroyed However, even with the best preparations it is not possible to obtain stoichiometric quantities of any product In the absence of added DPN and aldehyde dehydrogenase less than 5% of the 5HIAA appears Even with these components added less than 60% of the 5HIAA appears when mitochondria are incubated with serotonin There have been many attempts to solubilize mitochondria in order to purify the components of amine metabolism However this has not as yet been successful Fortunately some tissues, when homogenized, yield appreciable amounts of MAO in the soluble form (Weissbach *et al* 1957b) This soluble MAO has been fractionated into two components amine oxidase and aldehyde dehydrogenase It was found that one atom of oxygen was utilized for each equivalent of serotonin metab

olized when the purified MAO fraction was incubated with serotonin MAO was coupled with aldehyde dehydrogenase, one equivalent o was reduced for each equivalent of serotonin metabolized and each alent of 5HIAA formed

Using the partially purified soluble MAO which had been separate aldehyde dehydrogenase, it was possible to obtain appreciable qu of the intermediate, 5 hydroxyindole 3 acetaldehyde, and to det some of its properties Contrary to expectations this aldehyde was fc be relatively stable when kept in dilute solution This same enzyme aration was used to prepare the corresponding aldehyde from norep rine, i e, 3,4 dihydroxyphenylglycolic aldehyde (Leeper *et al*, 1958 of interest that this aldehyde was also found to be surprisingly st dilute aqueous solution

Although the term monoamine oxidase suggests a single enzyme s it is now apparent that there are a variety of enzymes which oxid deaminate amines The classification into diamine oxidase and MAO not absolute since MAO will metabolize diamines including hist (Fouts *et al*, 1957) Amine oxidizing enzymes are also present in the of certain species (Tabor *et al*, 1954, Blaschko *et al*, 1958) These en are not like the mitochondrial MAO It may well be that there are number of amine oxidases, some of which are fairly specific for a amine and others quite nonspecific A similar situation exists with r to cholinesterase activity

Whether MAO is one enzyme or a myriad of them, it is now ap that certain compounds which inhibit the enzymatic oxidative deami of serotonin and other amines *in vitro* are capable of inhibiting this *in vivo* When such compounds are administered to animals or pa *levels of serotonin and other amines are increased in the tissues* The in in brain serotonin level is now widely used as an index of the ability o ious drugs to produce MAO inhibition *in vivo* in animals

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Whether MAO is one enzyme or a myriad of them, it is now apparent that certain compounds which inhibit the enzymatic oxidative deamination of serotonin and other amines *in vitro* are capable of inhibiting this action *in vivo* When such compounds are administered to animals or patients, levels of serotonin and other amines are increased in the tissues The increase in brain serotonin level is now widely used as an index of the ability of various drugs to produce MAO inhibition *in vivo* in animals

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ether and procaine amide are excellent inhibitors of MAO *in vitro* but are not very active when administered *in vivo*

Even where activity can be demonstrated in animals *in vivo* it is important to know whether a given compound exerts its action in humans in the dosage given to produce its characteristic pharmacologic or therapeutic effect A test based on the conversion of orally administered serotonin to 5HIAA has been devised by Sjoerdsma *et al* (1958) to measure MAO inhibition in man *in vivo* Although this test measures predominantly the MAO activity of intestine and possibly liver one can assume, where a drug is readily absorbed that the activity in other organs will be affected

TABLE II

EFFECT OF VARIOUS DRUGS ON MAO IN MAN AND *in Vitro*

Drugs	Dose (mg)/ interval (hr)	% Inhibition of the conversion of serotonin to 5HIAA in man	Conc producing approx 50% inhibition *in vitro*[b]
Harmaline	75/8	70–80	10^{-6} *M*
JB 516	25/24	70–80	5×10^{-6} *M*
Iproniazid	100/8	50–70	3×10^{-4} *M*
Procaine amide	500/6	25	10^{-4} *M*
Orthoxine	100/4	20	10^{-4} *M*
d Amphetamine	10/2	0	10^{-3} *M*
Ephedrine	100/2	0	$>10^{-3}$ *M*
Hydralazine	50/6	0	$>10^{-3}$ *M*

The data in the above table are taken from a paper presented at the New York Academy of Sciences meeting Nov 1958 by Dr Albert Sjoerdsma

[b] MAO activity measured by serotonin disappearance technique in rat liver homogenates

to a similar extent In fact, there is evidence that the MAO of brain may be even more sensitive to MAO inhibition that is that of liver (Hess *et al*, 1958 Horita, 1959) Comparison of MAO inhibition of some compounds *in vitro* and *in vivo* in man are presented in Table II

It is apparent that in general there is agreement between *in vitro* and *in vivo* activities The most potent inhibitors, harmaline (1 methyl 2 phenyl) ethyl hydrazine hydrocholoride (JB 516) and iproniazid produce marked diminution in conversion of serotonin to 5HIAA It is of interest that ephedrine and amphetamine, compounds which had in the past been considered as acting through MAO inhibition, do not possess any degree of activity *in vivo* Their activity *in vitro* is actually quite low since they have little inhibitory activity below 10^{-3} *M*

That MAO inhibitors markedly block metabolism of serotonin has been shown by the increase in brain levels of serotonin which appear following

their administration Studies of this type are now commonplace However, it is of interest that the serotonin content of peripheral tissues does not rise as markedly during MAO administration Furthermore, although MAO inhibitors potentiate the effects of parenterally administered serotonin to some extent, they have little effect on the disappearance of the amine from the tissues (Fig 2) (Weissbach *et al*, 1957b) This is so because of alternative routes of metabolism which are sufficiently potent to metabolize the serotonin in the absence of MAO at almost the same rate as in the normal animal (Weissbach *et al*, 1957c) In the mouse, rat, guinea pig, and

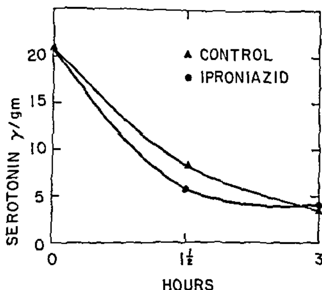


FIG 2 Effect of iproniazid on serotonin levels in whole mice after serotonin Mice were pretreated with iproniazid phosphate (300 mg /kg), and after 30 minutes received 80 mg /kg of serotonin Control animals received serotonin alone At various *time intervals the whole mice including excreta were assayed for serotonin* Each point represents three animals (Taken from Udenfriend, S , Weissbach H and Bogdanski D F 1957 *J Pharmacol Exptl Therap* **120** 255–260)

rabbit an alternative route of serotonin metabolism is conversion to serotonin O glucuronide (Weissbach *et al*, 1958) It is of interest that in mice where normally only 30% of an administered dose is excreted as 5HIAA, following administration of iproniazid the 5HIAA excretion is diminished to almost zero This is in agreement with the conclusions of Nakai (1958), who also suggested that where 5HIAA excretion does not balance serotonin ingestion it is owing to utilization of the primary product of MAO action, 5 hydroxyindoleacetaldehyde, for reactions other than 5HIAA formation, pigment, etc

Although glucuronide formation accounts for must of the serotonin metabolism following MAO inhibition in animals, it does not seem to occur to any great extent in man Although the extent of conversion of orally

administered serotonin to 5HIAA can be markedly inhibited, little serotonin penetrates into the blood stream, this suggests rapid metabolism by the gastrointestinal tract and liver The nature of these products remains to be determined (Sjoerdsma *et al*, 1958)

Urine and tissue extracts from carcinoid patients have revealed the presence of metabolites other than 5HIAA Thus a small but definite amount of conjugated 5HIAA has been demonstrated by Curzon (1955) This is presumably the *O* sulfate ester Chadwick and Wilkinson (1958) have also reported the formation of serotonin *O* sulfate by rat liver homogenates McIsaac and Page (1958) have reported the presence of *N* acetyl serotonin as well as several unidentified products in carcinoid urine

Another route of serotonin metabolism which obviously occurs in many amphibia (Erspamer, 1954b) and in plants (Stromberg, 1954 Wieland *et al*, 1953) is *N* methylation, to yield *N* methylserotonin, bufotenine, and bufotenidine Reports of *N* methylated products of serotonin in human urine (Bumpus and Page, 1955) have not been confirmed (Rodnight, 1956) In fact, in carcinoid urine, where all other metabolites of serotonin are markedly increased no trace of bufotenine could be detected (Sjoerdsma *et al*, 1957)

Some interesting studies on the bacterial metabolism of serotonin and other 5 hydroxyindoles have been reported by Oginsky *et al* (1958) Several microorganisms, including *Alcaligenes faecalis* and *Aerobacter aerogenes*, were shown to oxidize these compounds, and induced enzyme formation has actually been demonstrated However the significance of these findings to the physiology of the microorganisms is not evident as endogenous 5 hydroxyindoles have not been demonstrated As these are fecal microorganisms, it has been suggested that some 5 hydroxyindole metabolism may occur in the gut Although there is little evidence for this, it must still be considered a possibility A summary of the various routes of serotonin metabolism is shown in Fig 3

VIII Turnover of Serotonin in Tissues

The catalysts involved in serotonin formation and destruction appear to be widely distributed in tissues They are present not only in tissues which are rich in serotonin, but also in those which are found to contain little of the amine Under such conditions the amount of serotonin found in a tissue is not necessarily a reflection of its ability to synthesize and metabolize it In fact, studies by Udenfriend and Weissbach (1958) on the turnover of serotonin in tissues indicate large differences from tissue to tissue Thus the serotonin in the gastrointestinal tract in the rabbit has a half life of 10–17 hours whereas serotonin in the platelet has a half life of about 33 hours Surprisingly, the brain depots of serotonin which are the smallest, turn over

most rapidly Estimates of the half life of brain serotonin, based on the rapidity with which MAO inhibitors produce an increase, indicate that this is of the order of minutes (Udenfriend and Weissbach, 1958, Brodie *et al*, 1958) This rapid synthesis and metabolism of serotonin cannot be without

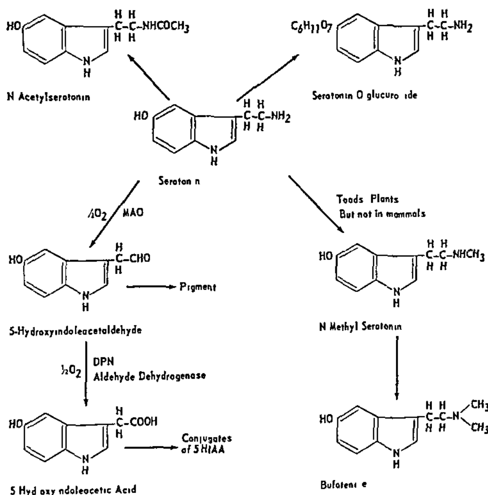


FIG 3 Routes of serotonin metabolism

significance with respect to the function of this amine in the central nervous system

IX LOCALIZATION OF IN ANI S

It should be pointed out that otonin is the blood and in the brain, the bulk of body, p 5%, is in the mucosa of stromic i emen n and its metabolites n, there le value changes in the meta otonin brain has wide n c

method to psychiatric and behavioral studies Unfortunately the major finding of such studies to date has been the detection of serotonin in bananas (Anderson *et al* 1958 Waalkes *et al* 1958)

Much of the serotonin in intestine and brain is localized in particulate material A preliminary report by Walaszek and Abood (1957) indicates that it may be localized in mitochondria Blaschko (in press) has recently reported similar findings However other reports indicate that studies on the intracellular localization of serotonin are difficult to carry out and interpret particularly in brain (Bogdanski *et al* 1957)

Another tissue which contains appreciable amounts of serotonin is the blood platelet In blood that has been carefully collected essentially all the serotonin is found in the platelets This finding has been the subject of much investigation Since platelets do not contain enzymes for making or metabolizing serotonin, it is apparent that they must take up serotonin which is released from tissues It is generally agreed that platelets, *in vitro*, can bind additional serotonin However, reports on the extent of *in vitro* binding seem to differ from laboratory to laboratory Thus Humphrey and Toh (1954) reported that platelets bind only small amounts of additional serotonin and that they may be saturated with respect to this substance However Bogdanski *et al* (1957) using higher concentrations of serotonin in the suspending fluid (over 1 mg /ml) were able to obtain a five to tenfold increase in platelet bound serotonin Although concentrations of 1 mg /ml are never encountered in tissues it should be pointed out that when serotonin is infused into an intact animal the serotonin content of platelets can be increased as much as twenty fold when the plasma level does not exceed 2–3 μg /ml during the entire period of the infusion Furthermore, as shown in Table III elevation in platelet serotonin level brought about by serotonin infusion persists for many days It would appear from the studies on binding and from the enzymatic and turnover studies on platelets that serotonin is taken up by the platelets as the amine is released from the tissues The serotonin is then held by the platelets until they rupture whereupon it is released The rate of turnover of platelet serotonin is then a reflection of the turnover of platelets themselves

The nature of the binding of serotonin by platelets has been the subject of much study too Hillarp *et al* (1955) suggested that adenosine triphosphate (ATP) may be involved in the binding of serotonin in platelets Born *et al* (1958) report the presence of large amounts of ATP in the platelets and consider the possibility of a direct combination of serotonin and ATP The exact nature of such a combination is not clear especially since the amounts of serotonin and ATP are not equivalent However, it should be pointed out that the chromaffin granules of the adrenal medulla are also rich in ATP and bind another group of amines, norepinephrine and epi

of urinary 5HIAA have been reported in other clinical manifestations, a urinary excretion of over 25 mg of 5HIAA per day has been reported only in malignant carcinoid Normal values range from 3–10 mg per day, in carcinoid patients levels of over 1000 mg per day have been reported but 200–400 mg per day are common Exogenous sources of serotonin, such as bananas and other fruits, should be considered, but their contribution to a 24 hour urine specimen is normally insufficient to prove misleading A casual specimen may, however, give significant elevations even after ingestion of only one banana

Recently two cases have been reported in which 5HIAA excretions were very high and in neither case was evidence for carcinoid obtained (McMullen and Hanson, 1958) In one case, laparotomy did not reveal a tumor In the second, liver metastases were isolated but pathologists classified them as carcinoma by histological techniques This report points up a very important fact that pathologists have neglected The tumor classified as carcinoid contains and secretes serotonin It is the reviewer's contention that detection of serotonin in biopsy or autopsy material is a simpler and more valid criterion for carcinoid identification than are the various histological methods There is no reason why suspected carcinoids should not be subjected to chemical analysis [4] They would then be classified as serotonin secreting tumors or non serotonin secreting tumors, which may be an even more significant classification than carcinoid and noncarcinoid In fact, all tumors which are known to secret specific substances should be subjected to chemical assay, at least for confirmation of the pathological findings

An interesting finding is that several patients with malignant carcinoid have been reported to secrete mainly the precursor of serotonin, 5HTP The significance of this finding and just how such patients differ from those with the usual serotonin secreting carcinoid remains to be determined Chromatographic study of carcinoid urine can aid in the detection of such 5HTP tumors

Another area which has received lots of attention is hypersensitivity The current status of serotonin in this field may be summarized as follows Serotonin is released from some *animal tissues during anaphylactic shock* and has been implicated in various antigen antibody interactions in animals Serotonin has also been found in the mast cells of rats and mice However, from a clinical standpoint changes in 5 hydroxyindole metabolism have yet to be implicated in any aspect of hypersensitivity The absence of sero

[4] Unfortunately tissues that are preserved in formaldehyde cannot be used for serotonin assay However the serotonin is stable indefinitely when tissues are stored in the deep freeze

tonin from human skin and mast cells rules out all the interesting observations made on rats

The sensitivity of the isolated uterus to serotonin made obvious the suggestion that it should be investigated in obstetrics However, the rat uterus *in situ* is not at all sensitive to serotonin Enormous quantities of serotonin in the uterus do not bring on contractions even during the later stages of gestation (Davidson *et al*, 1957) There is little evidence of a serotonin involvement in the function or pathogenesis of the human uterus

The bulk of the serotonin in the body is found in the gastrointestinal tract The gut fraction has been estimated as from 90 to 95% It is obvious, therefore, that significant changes in 5 hydroxyindole metabolism should be detectable in some clinical manifestations involving the gastrointestinal tract There have been reports that urinary excretion of 5HIAA is elevated (less than twofold) in patients with sprue (Kowlessar *et al*, 1958) This may signify hypersecretion from the mucosa It is of interest that following radical resection of the gastrointestinal tract in a patient the urinary 5HIAA levels fell to negligible values (Weissbach *et al*, 1959) It may be that further investigation will lead to interesting findings relating 5 hydroxyindole chemistry to the function and pathology of the gastrointestinal tract

Since serotonin does affect the cardiovascular system when administered parenterally it has been considered as a possible factor in hypertension However, there is no evidence of a 5 hydroxyindole abnormality in any clinical condition involving hypertension or hypotension

The central aspects of serotonin are of course most interesting to consider, as there have been many interesting laboratory findings concerning serotonin in brain However as stated earlier, evidence of a 5 hydroxyindole abnormality in any type of psychiatric disorder has yet to be satisfactorily demonstrated Although no one doubts that serotonin is present in brain and that it must play some important role there, attempts to relate abnormal behavior and the actions of various centrally acting drugs in man to specific changes in 5 hydroxyindole chemistry have not been too fruitful

Of all the drugs used only the Rauwolfia alkaloids and MAO inhibitors can be shown to influence serotonin metabolism or disposition in man Measurements of circulating serotonin following reserpine can be useful in determining the persistence of its effects in patients (Haverback *et al*, 1957) On the other hand although changes in circulating serotonin or urinary 5HIAA do occur following administration of MAO inhibitors, their magnitude is so small as to require statistical treatment of the data (Shore *et al* 1958) In the case of MAO inhibitors it has been found that after their administration to patients the urinary excretion of many other amines such as tryptamine, are increased to a much greater extent than is that of sero

tonin (Sjoerdsma et al, 1959) The urinary excretion of tryptamine has been suggested as a good index of MAO inhibition in man, superior in many ways to 5 hydroxyindole measurement Of course these other amines must also be considered in the over all interpretation of the effects of MAO inhibitors

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Ergothioneine*

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I Introduction

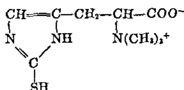
Ergothioneine is a substance which has intrigued biochemists for half a century Originally discovered in a fungus, it was later found in the red blood cells of animals Through the years, attempts have been made to cast light on what are its origin, function, and fate The answers to these questions proved to be particularly elusive In recent years, owing chiefly to improved methods of analysis and the application of isotopic tracer techniques, much progress has been made It seems likely, too, that the currently expanding interest in the compound may soon resolve some of the still unanswered questions The time is opportune, therefore, to present a brief review of the accumulated knowledge concerning ergothioneine

Although several biological activities have been suggested for ergothioneine in the past few years, the full meaning of these activities is not yet clear It is quite likely that its chief function or functions have not yet been uncovered Thus it is obviously not yet possible to classify ergothioneine in the realm of biologically active compounds such as vitamins or hormones It now appears fairly certain that ergothioneine is not synthesized by higher animals On the other hand, experiments so far have failed to demonstrate that animals need the compound for survival under normal laboratory conditions At the present state of knowledge it would appear that ergothioneine cannot be classified as a vitamin in the usual sense of the term On the other hand, recent developments have suggested that ergothioneine may have regulatory effects on enzyme activity In addition, a possibility which has not yet been explored is the effect of ergothioneine on animals under various stress conditions It is within the realm of possibility that the substance cannot be neatly categorized in any of the recognized classes of biologically active compounds, indeed, it is perhaps for this reason that its function or functions have defied elucidation for so long

II Historical

During the course of studies on ergot, the fungus infection of rye grain, Tanret (1909) isolated a crystalline, sulfur containing compound to which he gave the name *ergothionéine* The empirical formula was determined as $C_9H_{15}O_2N_3S$ The substance was optically active, and on the basis of yield was present to the extent of 0 1% in ergot Soon thereafter Barger and Ewins (1911) studied the chemical structure of the compound and from color reactions and chemical degradations, suggested that it was the betaine

of a new amino acid, 2-mercaptohistidine ("thiolhistidine") Later work has amply confirmed the correctness of the structure they proposed



Ergothioneine

For several years following its discovery, little attention was paid to ergothioneine Then in the early 1920's two independent groups of workers, led by G Hunter in Toronto and S R Benedict in New York, isolated from blood a substance which caused erroneously high values in the determination of uric acid (Hunter and Eagles, 1925, Benedict *et al*, 1926) This substance, which was named *sympectothion* by Hunter and *thiasine* by Benedict, was soon shown to be identical with ergothioneine from ergot (Newton *et al*, 1927, Eagles and Johnson, 1927) Because of its widespread occurrence in animals, Benedict suggested that the name *ergothioneine* be modified to *thioneine* This term suffers from the possibility of confusion with the organic dye *thionine* and has not been used by most workers in the field However, it has persisted in many textbooks and is used by *Chemical Abstracts* In the light of recent developments which strongly suggest that ergothioneine is wholly fungal in origin, it would appear that the name *ergothioneine* is more appropriate Should a more manageable term be desired to replace the cumbersome and frequently misspelled *ergothioneine*, the name *ergothione* should prove satisfactory from an indexing viewpoint, and at the same time would parallel more closely *glutathione*, another sulfhydryl compound with interesting similarities

The discovery of ergothioneine in blood stimulated considerable interest in the compound and in succeeding years several papers were published An important contribution was the development by Hunter (1928) of a sensitive and highly specific quantitative test for ergothioneine based on the coupling of ergothioneine with diazotized sulfanilic acid With this method Hunter showed that ergothioneine was widely distributed in the red blood cells of animal species

The discovery of the widespread occurrence of ergothioneine directed attention to its origin One of the earliest pieces of evidence bearing on this was published by Eagles and Vars (1928) They found that the blood ergothioneine level of the pig which possesses the highest level of any animal species, was markedly influenced by diet Although grain fed animals showed normal levels pigs which had been fed garbage or a purified casein diet had no detectable ergothioneine in their blood The inclusion of corn in the diet of these pigs led to the appearance of ergothioneine in the

blood An acid hydrolyzate of the corn protein zein gave a color with the Hunter diazo test which these authors interpreted as being due to the presence of a precursor of ergothioneine, and they suggested thiolhistidine as the precursor Further evidence for the effect of diet on blood ergothioneine was later provided by Potter and Franke (1935), who studied the effect of several cereal grains It was found that the feeding of oats, corn, or wheat to rats led to appreciable blood ergothioneine levels, while barley and potatoes gave extremely low levels

The effect of diet on blood ergothioneine levels has been confirmed by more recent work However, the suggestion by Eagles and Vars that thiolhistidine is a precursor of blood ergothioneine was based on extremely tenuous data, which in view of later developments appear to have been interpreted incorrectly Nevertheless, the suggestion had appreciable influence on subsequent thinking, even to the extent that thiolhistidine has been included in some textbooks as a constituent of proteins On the basis of available data, there is no evidence to show that thiolhistidine occurs in nature or is in any way connected biologically with ergothioneine

Ever since the original discovery of ergothioneine, attempts have been made to ascertain its biological significance Barger and Ewins (1911) mentioned in passing that the substance has no marked physiological action Later pharmacological studies by Tainter (1927) and Trabucchi (1936) confirmed its relative inertness Eagles and Cox (1928) showed that it could not replace the histidine requirement of growing rats Several years later considerable interest in ergothioneine and its relationship to the thyroid gland was evoked by the finding of Lawson and Rimington (1947) that ergothioneine possessed antithyroid activity comparable to that of thiouracil However, Astwood and Stanley (1947) and others were unable to obtain this effect, and the original results have never been satisfactorily explained

As may be gathered from the foregoing brief historical review knowledge concerning the biochemistry of ergothioneine accumulated extremely slowly during the four decades following its discovery in ergot In the last decade a renewed interest in ergothioneine has led to an accelerated accumulation of knowledge about its chemistry and physiology These more recent developments, together with a fuller discussion of some of the earlier articles, will be presented in the following sections

III ISOLATION

Ergothioneine has been isolated from microbial, plant, and animal sources Although the compound has also been obtained by laboratory synthesis, isolation is still the method of choice for preparative purposes The most frequently used starting materials have been ergot and red blood

cells. Because of the characteristically high levels found in pigs' blood from this animal is preferable to other bloods. However, pig blood, although high in comparison with the blood from other animals, contains levels appreciably less than those found in ergot. Hunter *et al.* (1949a) analyzed ergot samples from several different grain sources and found the ergothioneine content to range between 180 and 530 mg. per 100 gm. of dried ergot; values given for pig blood by Hunter (1928) vary from 4 to 53 mg. per 100 ml. of cells. The discovery that ergothioneine is synthesized by common fungi (Melville *et al.*, 1956) suggests a promising source of ergothioneine as a by-product of the large-scale cultivation of molds which forms the basis for the commercial production of penicillin, citric acid, and other chemicals.

1. Isolation from Ergot

The preparation of ergothioneine is most conveniently accomplished by the use of ergot as the starting material. Tanret (1909) used an alcoholic extract of ergot; after intermediate purification steps, the ergothioneine was separated as the insoluble mercury salt, which was then regenerated to give the crystalline hydrochloride of ergothioneine. He obtained a yield of 0.1% based on the weight of the ergot. A similar procedure was used by Eagles (1928) with intermediate precipitation of impurities with barium hydroxide and with basic lead acetate. The ergothioneine was then precipitated with mercury and finally with phosphotungstic acid. The yield of free base was 0.065%. Pirie (1933a) used a simplified procedure which took advantage of the formation of an insoluble copper complex of ergothioneine, as first described by Williamson and Meldrum (1932) in the preparation of ergothioneine from blood. Pirie's procedure involved the removal of impurities from an aqueous extract of ergot with basic lead acetate, precipitation of ergothioneine by the addition of cuprous oxide, and regeneration of the copper complex with hydrogen sulfide. The yield of crystalline ergothioneine hydrochloride was 0.18%. Hunter *et al.* (1949b) modified the procedure of Pirie by using uranium acetate in place of lead acetate to remove impurities; a high yield of ergothioneine (0.26%) was obtained. Comparable yields have been experienced in the author's laboratory by using Pirie's procedure. It seems likely that the variation in yields experienced by different workers is a reflection in large part of variations in the ergothioneine content of different ergot samples.

2. Isolation from Blood

The isolation of ergothioneine from blood was first accomplished several years after the isolation from ergot. However, it was not suspected at the time that ergothioneine occurred in blood; the procedures used were directed

toward the isolation of the unknown substance which interfered with the determination of blood uric acid (Bulmer *et al*, 1925, Benedict, 1925) The first published procedure for its isolation was described by Hunter and Eagles (1925), although Benedict *et al* (1926) stated that it had been isolated in their laboratory some five years earlier The procedure of Hunter and Eagles (1925) used red cells of the pig as starting material and involved purification steps with uranium acetate, lead acetate, mercuric chloride, and phosphotungstic acid Benedict *et al* (1926) also used pig blood but purified the ergothioneine by precipitation with silver lactate and then with mercuric chloride Both procedures gave comparable yields, within the range of 60 to 100 mg of ergothioneine per liter of blood Later, Williamson and Meldrum (1932) introduced the use of the insoluble copper complex of ergothioneine and thereby simplified the isolation procedure In their method, the protein free blood filtrate is first aerated to oxidize glutathione to the disulfide form and thereby prevent its subsequent precipitation with copper and then the copper complex of ergothioneine is precipitated by the addition of cuprous oxide After regeneration from the copper salt, ergothioneine is obtained in an amount equivalent to 85% of that present in the blood as determined by the Hunter diazo reaction

3 Isolation from Other Sources

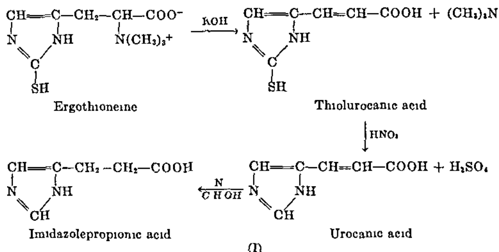
The isolation of ergothioneine from several sources other than ergot and pig blood has been accomplished, primarily for the purpose of proving the presence of ergothioneine in the source materials Successful isolations have been reported from human blood (Newton *et al*, 1927) and human urine (Sullivan and Hess, 1933) pigeon blood (Gulland and Peters, 1930), boar semen (Leone and Mann, 1951, Heath *et al*, 1957), oats (Melville and Eich, 1956), and *Neurospora crassa* (Melville *et al*, 1956) The paper by Heath *et al* describes the use of ion exchange chromatography for the purification of ergothioneine, and crystallization of the compound as the tartrate, while the papers by Melville and co workers illustrate the use of chromatography on alumina Difficulties with ion exchange resins apparently leading to structural changes in the ergothioneine molecule have been mentioned by Grossman and Kaplan (1958)

IV Chemistry

1 Structure

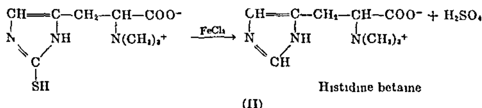
a Degradation Tanret (1909) isolated ergothioneine as a crystalline dihydrate for which he established the empirical formula $C_9H_{15}O_2N_3S \cdot 2H_2O$ He also obtained and characterized the hydrochloride, sulfate phosphate, and the silver and mercury salts Barger and Ewins (1911) found that

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The second degradative reaction was brought about by the action of hot ferric chloride solution From this reaction (II) a sulfur free base, $C_9H_{15}O_2N_3$, was isolated as the dipicrate Barger and Ewins suggested that this substance was the betaine of histidine, formed by the oxidative removal of the sulfur atom of ergothioneine



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It is interesting that in the same year Kutscher (1911) described the isolation from Hercynia a commercial extract of mushrooms of a new base which he characterized as a trimethyl derivative of histidine This compound was later given the name *hercynine* and was shown to be identical with the oxidation product from ergothioneine

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3 *Isolation from Other Sources*

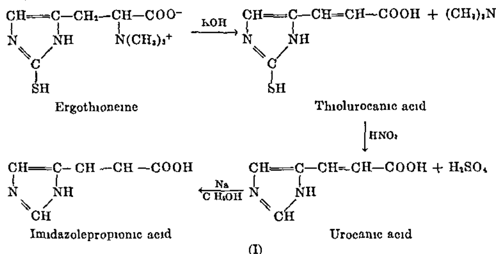
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IV CHEMISTRY

1 *Structure*

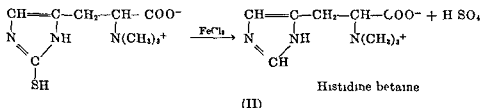
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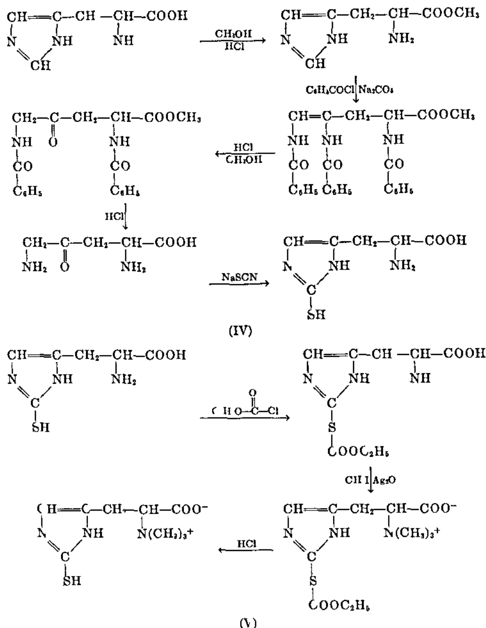
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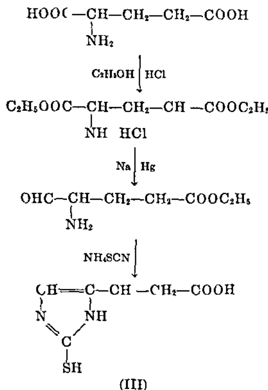


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2 Isotopically Labeled Ergothioneine

The above synthesis (V) can be used for the preparation of ergothioneine labeled with isotopes Sunko and Wolf (1958) prepared DL-ergothioneine

The data of Barger and Ewins did not permit definite allocation of the position of the sulfur atom in ergothioneine, but the stability of the sulfur toward alkali and its ready oxidation by ferric chloride or bromine water suggested the presence of a 2 mercaptoimidazole ("thiolimidazole") structure Confirmation of this was provided by Akabori (1933) who reduced the alkali degradation product (thiolurocanic acid) with sodium amalgam to give β 2 mercaptoimidazole 4(5) propionic acid The structure of this compound was confirmed by synthesis (III) from glutamic acid

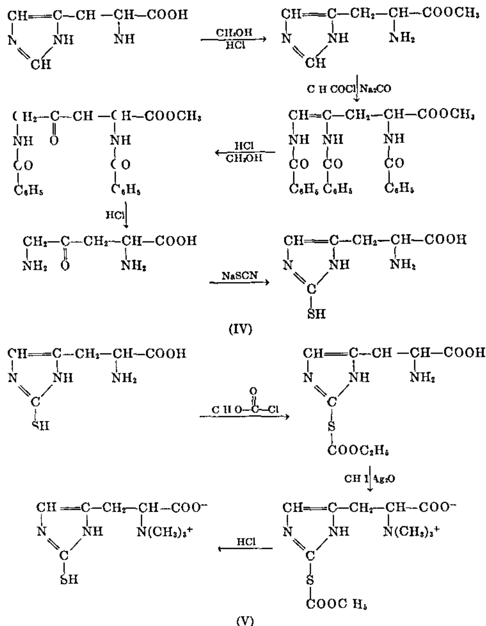


(III)

b Synthesis The laboratory synthesis of ergothioneine has proved to be more difficult than might be expected from its relatively simple structure Harington and Overhoff (1933) and Jackson and Marvel (1933) have recorded unsuccessful attempts The compound was finally synthesized by Heath *et al* (1951a) from thiolhistidine (2 mercaptohistidine) This amino acid has been prepared by several methods A convenient synthesis is the procedure (IV) of Ashley and Harington (1930) starting with histidine

Optically active thiolhistidine is obtained from L histidine For the synthesis (V) of ergothioneine, Heath and co workers first protected the sulfur of L thiolhistidine with a carbethoxy group, and then methylated the α amino group with methyl iodide The carbethoxy group was subsequently removed by acid hydrolysis

The synthetic material gave correct analyses and behaved identically to ergothioneine with respect to color reactions precipitating agents, paper



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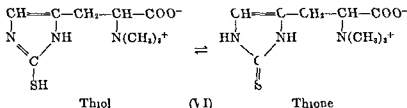
a Physical Ergothioneine is a colorless, odorless substance which is moderately soluble in cold water, very soluble in hot water, only slightly soluble in ethanol and insoluble in nonpolar solvents It crystallizes as platelets of the dihydrate from water, and as needles or prisms of the anhydrous form from aqueous ethanol The anhydrous material melts with decomposition at 256–257°C (corrected) in the capillary tube and chars but does not melt up to 300° on the micro stage The optical activity of aqueous solutions has been given as $[\alpha]_D$ +110° (Tanret, 1909) and $[\alpha]_D^1$ +116° (Newton *et al*, 1927) The configuration of the α carbon atom is apparently the same as the L series of amino acids, since the synthetic material, *although showing a lower optical activity, is also dextrorotatory* The ultraviolet absorption spectrum in water displays a single peak at 257–258 mμ (Holiday, 1930, Heath *et al*, 1951a) and a molar absorptivity of approximately 16,000 The higher absorption given by Holiday is ap parently in error The effect of pH on the ultraviolet absorption has been studied (Heath and Toennies, 1958) In 95% ethanol the peak is shifted to 264 mμ (Melville *et al*, 1955b)

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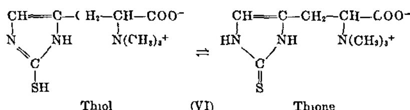
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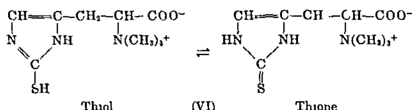
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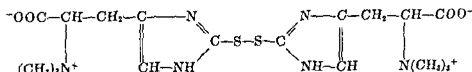
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typical reactions of aliphatic sulfhydryl compounds, for example, ergothioneine gives negative results in the nitroprusside test (Hunter and Eagles, 1925) and in the Sullivan test for cystine (Sullivan, 1929) It does however, react positively in the sodium azide iodine test for sulfur compounds (Friedmann, 1936), and adds to DPN (van Eys and Kaplan, 1957)

The presence of the sulfur atom also affects the basicity of the imidazole ring The normal basic properties of imidazoles are not apparent in the thiolimidazoles, in fact the ring system is somewhat acidic The presence of the quaternary ammonium group in ergothioneine is responsible for the formation of salts of ergothioneine, such as the chloroplatinate and phosphotungstate

The chemical reactivity of the sulfur atom of ergothioneine shows some marked differences when compared with the sulfur of cysteine or glutathione Although ergothioneine is comparatively stable toward hot mineral acid (Hunter and Eagles, 1925), the stability of the sulfur toward strong alkali is remarkable Under severe alkaline conditions which readily split sulfur from cysteine as inorganic sulfide, ergothioneine loses trimethylamine but the thiolimidazole portion of the molecule remains essentially intact, and thiolurocanic acid can be recovered in good yield (Barger and Ewins 1911) Perhaps even more remarkable is the sensitivity of the sulfur atom toward oxidizing agents This is exemplified by the ready formation of sulfuric acid when ergothioneine is treated with bromine water (Barger and Ewins, 1911) This property is characteristic of thiones but not of aliphatic sulfhydryl compounds such as cysteine and glutathione (Blumenthal and Clarke, 1935) Other oxidizing agents which have been shown to effect the same reaction include ferric ion, permanganate, and hydrogen peroxide

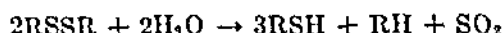
The ease with which the sulfur is oxidatively split from ergothioneine has made difficult the preparation of the disulfide form of the compound



Ergothioneine disulfide

Barger and Ewins (1911) mention that they obtained the disulfide by treatment of ergothioneine with iodine The product of this reaction was obtained as black, steel gray, or blue crystals which contained iodine However, no substantiating data were published Recently Heath and Toennies (1958) succeeded in preparing the disulfide It possesses some interesting chemical properties The compound was obtained as a deliquescent, yellow, amorphous dihydrochloride after oxidation of ergothioneine in $5N$ HCl with the theoretical amount of hydrogen peroxide The product is unstable,

omposing instantly in alkali and slowly in water, to give 60–70% of the
ount of ergothioneine which could be obtained by reduction It is probable
t the breakdown is similar to that observed by Balaban and King (1927)
a thiolimidazole disulfide which underwent hydrolytic fission of the
lfide bond in the presence of alkali, according to the following equation



$$2RSSR + 2H_2O \rightarrow 3RSH + RH + SO_2$$

n the case of ergothioneine disulfide the products would be ergothioneine,
cynine, and sulfur dioxide

Heath and Toennies found that ergothioneine disulfide was readily re
ced to ergothioneine by cysteine, with the concomitant formation of
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fide between ergothioneine and glutathione was obtained in similar
hion The reverse reaction, the reduction of glutathione disulfide by
othioneine did not occur No disulfide interchange between ergothio-
ne disulfide and cystine could be detected

Ergothioneine disulfide dihydrochloride is stable only in alcohol strongly
d solution, or as the dry salt Chromatography on ion exchange resin or
imina causes decomposition The compound gives a positive Hunter
izo test, with a color value 65–70% of that given by ergothioneine this
undoubtedly owing to hydrolytic breakdown to ergothioneine under the
nditions of the test These data show that it would be impossible to
late the disulfide from natural materials with the procedures used at
ent It is also evident that ergothioneine disulfide, in contrast to
tathione disulfide cannot accumulate to any significant extent in tissues
rthermore, ergothioneine is much more resistant to oxidation by peroxide
molecular oxygen at physiological pH's than in strong acid (as contrasted
glutathione which shows the reverse behavior) It may well be that the
ajor function of ergothioneine in animal tissues is not directly concerned
th oxidation reduction mechanisms It is now evident that the oxidation
luction potential of ergothioneine as determined by potentiometric
ration (Ryklan and Schmidt, 1944) may be of little value

The facile oxidation of ergothioneine by various reagents can lead to
terference in the quantitative determination of other compounds by
idative procedures The original discovery of ergothioneine in blood was
sed on its oxidation by the arsenophosphotungstic acid reagent used to
termine uric acid It is also oxidized by the Folin Wu alkaline copper
agent and thus interferes to a slight extent with the blood sugar deter
ination (Benedict and Newton, 1929c) The effect of ergothioneine on the
dometric determination of glutathione has been studied by Lucas and

King (1932) and Woodward and Fry (1932), who found that the presence of ergothioneine caused some error in the determination, but this is apparently small under the conditions which are most suitable for the determination of glutathione. The latter authors showed that the presence of glutathione catalyzes the oxidation of ergothioneine by iodine or ferricyanide. A similar catalytic effect of glutathione was noted by Benedict and Gottschall (1932) in the oxidation of ergothioneine with phosphomolybdic acid or cupric ion. In contrast, Pirie (1933b) showed that neither cysteine nor glutathione catalyzed the oxidation of ergothioneine by hydrogen peroxide, but ergothioneine was an effective catalyst for the oxidation of cysteine by hydrogen peroxide.

The possibility of interference by ergothioneine in the estimation of ascorbic acid should be stressed. For example, Mann and Leone (1953) found that almost all of the reducing activity of boar semen toward 2,6-dichlorophenolindophenol could be accounted for by the ergothioneine which was present. With the recognition that ergothioneine occurs normally in many tissues besides blood (Melville *et al.*, 1954), and in relatively high concentrations in some, the effect of its presence on ascorbic acid determinations by the dichlorophenolindophenol method must be kept in mind. The use of p-chloromercuribenzoate has been proposed by Owen and Iggo (1956) to minimize the effect of ergothioneine and other thiols.

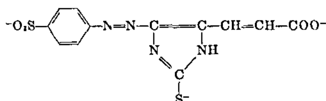
V. Methods of Determination

As with many other substances of biochemical interest, progress in the study of ergothioneine has been heavily dependent on the availability of adequate analytical methods. For many years after the discovery of ergothioneine in blood, the procedures which had been developed for its estimation were suitable for use only with blood, and they suffered from serious limitations even with blood. In recent years, blood methods have been improved, and, more importantly, chromatographic methods for the detection and quantitative determination of ergothioneine have been developed which are applicable not only to blood, but also to many other tissues and biological materials. These have been essential for studies on the occurrence, distribution, and biosynthesis of ergothioneine in various organisms.

1. *Quantitative Blood Methods*

The first method used for the quantitative determination of ergothioneine was based on its oxidation with arsenophosphotungstic acid, the reagent developed by Benedict for the determination of blood uric acid (Bulmer *et al.*, 1925; Benedict, 1925). A later modification (Behre and Benedict, 1929; Behre, 1932) gave considerably lower values because of improved specificity for ergothioneine. In this method, uric acid and ergo-

neine are precipitated from blood filtrates with silver, the uric acid is oved by extraction with HCl NaCl, and ergothioneine in the residue termined colorimetrically with the uric acid reagent An inherent dis ntage of the method is the nonspecific oxidizing nature of the reagent diazo procedure developed by Hunter (1928) has an advantage in this ect In this method, ergothioneine is coupled with diazotized sulfanilic and the yellow product is treated with strong alkali This causes the ation of a bluish red color by liberating trimethylamine from the ling product (Hunter, 1930) No other naturally occurring compounds known which react in this fashion to give the typical magenta color ined with ergothioneine (Lawson *et al*, 1951) Thiolurocanic acid gives same magenta colored compound as ergothioneine, but this occurs im iately on coupling with the diazo reagent, without the necessity for tment with alkali Lawson *et al* have proposed the following structure he colored product



olor formation does not occur when the sulfur atom carries an alkyl stituent The reaction with ergothioneine is very sensitive a detectable r is produced at a concentration of one part in 5 000,000 When applied nixtures such as blood the reaction suffers from the serious drawback many other substances (e g tyrosine and histidine) give yellow to nge products while some substances (e g cysteine) inhibit the color nation from ergothioneine itself The development of a satisfactory lytical method is therefore essentially a problem of removing interfering stances, without loss of ergothioneine, before application of the color ction

osses of ergothioneine in the precipitation of blood proteins have been erienced The usual tungstic acid reagent is unsatisfactory because of (Benedict *et al*, 1926) Molybdic acid (Benedict and Newton, 1929a, re and Benedict, 1929 Hunter 1949) and tungstomolybdic acid (Bene and Newton, 1929b, Salt 1931) give better recoveries More recent hods have used heat coagulation in acid solution (Hunter, 1949), or cipitation with uranium nitrate (Touster, 1951) or trichloroacetic acid elville and Lubschez 1953), for the removal of proteins

Because of its inherent advantages, the Hunter diazo reaction has been d more extensively than any other method However, the original cedure (Hunter, 1928) does not always give satisfactory results par

ticularly with blood samples containing low levels of ergothioneine Attempts to make the reaction more universally applicable to all kinds of bloods have resulted in the publication of several modified procedures Hunter (1949) published an improved procedure in which basic lead acetate is used to remove interfering substances, while Lawson *et al* (1950) precipitated ergothioneine with iodobismuthous acid prior to color development Lawson *et al* reduced interference by using red cells rather than whole blood, and Fraser (1951) used washed cells This is permissible since no ergothioneine can be detected in plasma and none is lost in washing the cells Early in the author's studies on ergothioneine, experiences with various methods revealed unsatisfactory results with some blood samples, so a new procedure was devised (Melville and Lubschez, 1953) In this procedure, washed cells are lysed and treated with glutathione and hydrosulfite, proteins are precipitated with trichloroacetic acid, and interfering substances are removed from the filtrate by extraction with chloroform and absorption on an ion exchange resin The Hunter diazo reaction in modified form is carried out on the resulting solution All bloods tested, except avian blood, give colors characteristic of pure ergothioneine with excellent recoveries and increased sensitivity

Litner (1948) described in a preliminary communication the application of the diazo reaction after the treatment of ultrafiltrates of blood or plasma with strong alkali Touster (1951) made use of the reaction of ergothioneine with bromine water to form inorganic sulfate, which was determined colorimetrically Quantitative recoveries were not obtained Ohara *et al* (1952) determined the amount of sulfate produced from blood and tissues by oxidation with alkaline peroxide Jocelyn (1958) has determined free and "bound" ergothioneine in blood and proteins by a method based on the estimation of trimethylamine released by heating the material with strong alkali The validity of results obtained with these methods cannot be assessed at this time since the absence of interfering substances has not, in the reviewer's opinion, been adequately demonstrated

It should be pointed out that all of the methods discussed so far may, in some instances, give values which are erroneously high Baldridge and Lewis (1953) have obtained evidence for the presence in blood cells of a substance, other than ergothioneine, which gives a red color in the diazo test Furthermore, a comparison of results from the direct method of Melville and Lubschez with those obtained by a more sensitive and specific chromatographic procedure (see below) showed that in the case of several rat blood samples the ergothioneine content by the chromatographic procedure was only 50–70% of that obtained by the direct method

In spite of the remaining inaccuracies and limitations of the direct methods, they are in general quite suitable for routine analytical work for

many kinds of studies A comparison of the methods of Hunter, Lawson *et al*, and Melville and Lubschez shows reasonable correlation The column chromatographic procedure, while giving more reliable results, is not as suitable for numerous routine analyses

2 General Quantitative Methods

Attempts to apply the blood methods to tissue analysis usually fail because of incomplete separation of interfering substances Heath *et al* (1952), in determining ergothioneine in rat tissues by the iodobismuthous acid method found it necessary to use as 'blanks' the tissues from animals presumably free of ergothioneine Several tissues were examined by Ohara and associates for alkaline peroxide labile sulfur, but it seems doubtful that the results represent true ergothioneine values Melville and Lubschez (1953) modified their blood method to permit analysis of tissues, but satisfactory results were obtained with only a few tissues The amperometric titration of ergothioneine has been described (Benesch and Benesch, 1948) but is not sufficiently sensitive Because of the obvious need for a more universal method, considerable time was spent in the author's laboratory on this problem The method which was devised is essentially one of chromatography on an alumina column, with a water ethanol formic acid mixture as the developing solvent (Melville and Horner, 1953 Melville *et al*, 1954) Fractions are collected automatically and ergothioneine is estimated by means of the modified Hunter diazo reaction (Melville and Lubschez, 1953) The method is satisfactory for the analysis of blood and most tissues as well as other biological materials A five to tenfold increase in sensitivity over the direct blood methods is possible, permitting determinations of levels as low as 0 05 mg per 100 gm of tissue There is also greater certainty that only ergothioneine is being measured When interfering material is bothersome, it is frequently possible to separate it by modifying the alcohol or acid content of the developing solvent A typical chromatogram showing the separation of ergothioneine from the amino acids of rat liver, is shown in Fig 1

3 Qualitative Methods

Paper chromatography has proved a convenient tool for the detection of ergothioneine in a variety of biological materials Instances of its application to ergothioneine can be found in papers by Work (1949) Woolf (1949) Lawson *et al* (1950), Ames and Mitchell (1952), Baldridge and Lewis (1953) Heath (1953), Mann and Leone (1953), and Mackenzie and Mackenzie (1957) The last authors make the interesting observation that ergothioneine appears to undergo chemical change in the electrolytic desalting procedure frequently used prior to chromatography For visualiza

tion of ergothioneine on paper, most workers have used the Hunter diazo reaction, preferably in conjunction with other color tests Some of the reagents which have been used include the Folin Marenzi reagent (Lawson *et al*, 1950), the Pauly reagent (Ames and Mitchell, 1952), 2,6 dichloro quinone chloroimide (Heath, 1953), 2,6 dichlorophenolindophenol, phosphotungstic acid, ammoniacal silver, ultraviolet absorption (Mann and Leone, 1953), and the sodium azide iodine reagent (Smith and Tuller, 1955) Benesch *et al* (1956) used *N* ethylmaleimide to form a pink color with ergothioneine and other sulfur compounds in nonaqueous, alkaline solution, but Alexander (1958) found that ergothioneine did not react in neutral, aqueous solution

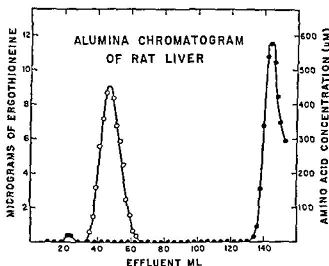


FIG 1 Distribution of ergothioneine (○) and ninhydrin reacting material (●) in alumina chromatogram of an extract of perfused rat liver Column solvent 1% citric acid in 75% aqueous ethanol

VI ANIMAL ERGOTHIONEINE

1 *Distribution*

Several investigations have pointed to the ubiquitous nature of ergothioneine in animals The work of Bulmer *et al* (1925), Hunter (1928), and Christman *et al* (1944) demonstrated its presence in the red cells of a variety of mammals It also occurs in birds (Hunter, 1928, Gulland and Peters, 1930), and has recently been identified as a constituent of the marine crab, *Limulus polyphemus* (Ackermann and List, 1958) In higher animals, ergothioneine has been found in the bone marrow of the human (Fraser, 1951) and the rat (Lutwak Mann, 1952), and in surprisingly high concentrations in the seminal vesicular secretion of the pig (Mann and Leone, 1953) and the ampullar secretion of the horse (Mann *et al*, 1956)

The presence of ergothioneine in human urine has been studied, but the results were variable Sullivan and Hess (1933) reported the isolation of the compound from urine, and Work (1949) detected it by paper chromatography However, Woolf (1949) and Lawson *et al* (1950) could find none It seems likely that the conflicting results can be explained by individual variations in the amounts excreted

Until recently, the lack of suitable analytical methods precluded definitive systematic studies on body tissues Heath *et al* (1952) examined the liver, kidney, and spleen of the rat, but found no ergothioneine except in animals to which the compound had been administered by feeding or by injection After the administration of S^{35} ergothioneine to rats, Heath (1953) found radioactivity in many tissues Melville *et al* (1954) applied their

TABLE I
Ergothioneine Levels in Animal Tissues

Tissue	Ergothioneine				Tissue	Ergothioneine
	Rat	Rabbit	Dog	Cat		Rat
Liver	13 3	0 3	0 9	2 7	Skeletal muscle	0 7
Blood cells	10 4	10 0	6 6	2 9	Intestines	0 6
Kidneys	4 3	0 3	1 6	3 1	Stomach	0 4
Heart	1 5	2 7	8 9	0 0	Seminal vesicles	0 0
Lungs	1 5	0 3	0 6	0 8	Brain	0 0
Spleen	1 1	1 0	1 1	—	Blood plasma	0 0
Testes	0 0	0 1	0 0	0 0		

Values are expressed as milligrams per 100 gm fresh tissue

method of column chromatography to the tissues of rats which had been *maintained on stock laboratory diets and found ergothioneine to be a* normal constituent of many tissues Large variations in the concentration of ergothioneine among different tissues were observed with liver showing even higher levels than the blood cells Comparative studies on other species would be highly desirable M Exner and D Kartchner in the author's laboratory have examined a limited number of species with interesting results (Table I) These analyses were performed on tissues from animals which had been perfused until essentially blood free It can be seen that there is a marked variation in the distribution pattern among the different species Liver, which is characteristically rich in ergothioneine in the rat, shows very low levels in the rabbit and dog The difference between heart tissue of the cat and dog is striking Blood cells in all four animals contain appreciable quantities while some tissues contain uniformly low concentra

tions Further studies might well uncover a relationship between ergothioneine and metabolic patterns of the tissues

The intracellular distribution of ergothioneine was studied in the case of rat liver (Melville *et al*, 1954) It appears that essentially all of the determinable ergothioneine is associated with the cytoplasmic fraction of the cells and is present in unbound form Whether bound ergothioneine exists in body tissues is not definitely known Ohara *et al* (1952) found peroxide labile sulfur in serum, and Jocelyn (1958) obtained trimethylamine from plasma proteins and red cell proteins That these products come from ergothioneine in the proteins has not been proved Recent evidence (Ackermann *et al*, 1959) suggests that hercynine is present in blood, this compound also yields trimethylamine on treatment with alkali It is known that the diazo test gives negative results with plasma Melville *et al* (1956) have shown that commercial bovine albumin preparations contain ergothioneine, but this is an inconstant occurrence and can very likely be ascribed to hemolysis M Ludwig in the author's laboratory injected S^{35} ergothioneine into a rat, daily for 3 weeks and 9 days later examined the blood for radioactivity None was found in the plasma The red cells were highly radioactive, but on lysis and dialysis none remained with the cell protein It is obvious that exogenous ergothioneine must transitorily appear in the plasma during its assimilation, but clear cut evidence for the presence of ergothioneine bound to protein by other than nonspecific interactions is still lacking This important point deserves further study

2 *Physiological Variations*

Up to the present time, practically all studies of variations in ergothioneine levels have been limited to blood It has long been known that blood levels vary widely, not only among species, but also among individuals within a species Table II, compiled from Hunter (1928) and other sources illustrates these facts Some of the factors which control blood ergothioneine levels have been discovered, but others remain unknown

a Diet That diet can affect blood levels has been recognized for many years The work of Eagles and Vars (1928), showing that the inclusion of corn in the diet of pigs increased the ergothioneine content of the blood, was followed by the studies of Potter and Franke (1935) demonstrating the varying effectiveness of different cereal grains in producing this effect in rats Corn oats, and wheat caused increased levels over those found with barley or potato, although different samples of oats gave variable results In contrast, Hunter (1951) was unable to find any significant effect of corn in increasing the blood ergothioneine levels of either pigs or rats More surprisingly, Baldridge and Lewis (1953) could detect no ergothioneine at all in the blood of rats which had been fed oats or corn Melville *et al*

(1955b) were unable to show any effect with the corn protein, zein, but did demonstrate the appearance of low levels of ergothioneine in the blood of rats maintained on purified diets to which corn or an extract of corn had been added Baldridge and Lewis (1953) found that rabbits which were fed a diet of cabbage and oats developed high levels of ergothioneine in the blood, and Baldridge (1955) showed that this effect was due to the oats

Contrasted with these conflicting observations are the uniform results obtained by several groups showing that purified diets cause decreases in blood ergothioneine levels in pigs (Eagles and Vars 1928), rabbits (Spicer *et al*, 1951 Baldridge and Lewis, 1953, Baldridge, 1955), and rats (Melville *et al* 1954) The rats studied by Melville and associates had been maintained on casein containing diets for six months, chromatographic analysis failed to detect any ergothioneine either in the blood or in other tissues

TABLE II

NORMAL BLOOD ERGOTHIONEINE LEVELS

Species	Ergothioneine	Species	Ergothioneine
Man	1–4	Dog	3–6
Rat	1–6	Ox	0 5–2
Rabbit	1 10	Pig	3–27
Guinea pig	1–4	Sheep	2–6
Cat	0 5–2	Fowl	2–10

Values are expressed as milligrams per 100 ml whole blood

It is well established that dietary ergothioneine gives rise to blood ergothioneine (e g Spicer *et al* 1951) It is also known that concentrations in the blood are determined to a large extent by the amount of ergothioneine in the diet (Melville *et al* 1955b) However that other dietary factors are also involved is indicated by the finding that fortified zein diets permit more rapid uptake of ergothioneine by red cells than do casein diets The reason for this effect is not known

b Geography The work of Eagles and Vars (1928) suggests that geographical location may affect blood ergothioneine levels Hunter (1951) has championed this idea and has provided data to show differences in the blood levels of rats, pigs, and humans in Canada and England For example values for the human in Edmonton ranged between 2 5 and 9 5 mg per 100 ml of blood while in London the range was 1 8 to 4 4 It is interesting that Fraser (1950) studied a group of 94 individuals in Edmonton and found a range of 2 to 8 mg per 100 ml of blood In the author's experience blood levels determined on several dozen individuals in New York City

have varied between 1 5 and 4 0 mg per 100 ml of blood, a range significantly lower than the Canadian values obtained by Hunter and by Fraser

c Age Touster and Yarbro (1952) found higher blood ergothioneine levels in adult humans than in infants (5 4 vs 3 1 mg %) No significant differences in various adult age groups were observed by these workers or by Fraser (1950) Laboratory rats which are fed a stock diet after weaning show increasing levels in the blood for several weeks, after which a plateau is reached (Beatty, 1952) Studies on rats which were fed controlled amounts of ergothioneine show that the rate of increase and the final levels are determined to a large extent by the amount in the diet (Melville *et al*, 1955b) In these experiments, rats were first depleted by feeding a casein diet, and then ergothioneine was added to the diet at levels of 0 001, 0 005, and 0 01 % Animals on the 0 001 % diet showed barely detectable levels in the blood after 2 months, while those consuming the 0 01 % diet showed values of 3–4 mg % in 2 weeks, and 7–9 mg % in 6 weeks The 0 005 % diet gave intermediate increases which were similar to those found with stock diets

d Race Preliminary data suggest that race may have some influence on blood ergothioneine levels The work of Fraser (1951) on hospital patients indicates that Canadian Indians and Eskimos may have tendencies to show higher values than the white population The uniformity of the hospital diets mitigates against differences due to dietary regimen Similarly, Touster and Yarbro (1952) studied hospital patients and found a tendency for higher blood ergothioneine values in negro patients than in white patients

e Sex While Potter and Franke (1935) observed no significant differences in the blood ergothioneine levels of male and female rats, Hunter (1951) found slightly higher levels in females than in males (2 4 vs 1 9 mg %) Mackenzie and Mackenzie (1957), on the other hand, showed that the blood levels in female rats reached plateau values in 6 months, but the levels in male rats continued to rise, particularly during the age period of 12 to 18 months, until the male levels were twice those of the female (15 2 vs 7 5 mg %) Most interestingly, it was found that the administration of testosterone propionate to the females increased their blood level to that characteristic of the males Neither castration nor the administration of estradiol to the males affected the subsequent rise in blood levels That this sex linked difference does not apply to the human is suggested by the work of Salt (1931) and Fraser (1950), who detected no differences between the levels in men and women

f Other Factors Although Hunter (1951) obtained fairly uniform blood levels in rats and in pigs on diets containing corn, Melville *et al* (1955b) found rather wide variations in the blood levels of rats which were main

tained on a casein diet containing 0 005 % ergothioneine These levels, which varied from 2 to 8 mg %, could not be correlated with age, sex, food intake, or weight gain It would appear that additional, unknown factors are involved in regulating blood ergothioneine levels

Ling and Chow (1953) observed a small increase in blood ergothioneine in two pernicious anemia patients after the administration of vitamin B_{12}, along with a larger increase in reduced glutathione With rats, however, no effect of vitamin B_{1} on blood or liver ergothioneine could be detected (Register, 1954, Baldridge, 1955) Bartlett and Register (1953) studied the effect of restraint and a cold environment on blood and liver sulfhydryl compounds in rats Cold, or cold and restraint, produced a slight decrease in the blood ergothioneine levels of the animals, but no significant change in liver ergothioneine

3 *Variations in Disease*

Bulmer *et al* (1925) recorded the first possible connection of blood ergothioneine with a pathological condition when they noted the relatively high levels in cases of "nickel rash" among workers in a nickel refinery These high levels were also found in refinery workers who had not developed symptoms This interesting observation has apparently not been pursued further Not long afterward, Benedict *et al* (1926) stated that diabetic patients tended to show high blood ergothioneine values, while in nephritis the tendency was toward normal or unusually low values Salt (1931) found essentially normal levels in nephritis, but confirmed the tendency toward high levels in diabetes mellitus The analytical methods in use at that time were not highly specific However, the extensive study by Fraser (1950) of over a hundred diabetic patients has confirmed the earlier results His values show an average ergothioneine level of 9 6 mg per 100 ml of red blood cells for normal subjects, compared to 12 7 mg % for diabetics Diabetic levels may be normal or low in many cases but there is an increase in the frequency with which high or abnormally high levels occur Fraser was unable to correlate the high levels in diabetes with the duration or state of control of the disease, or with age sex, blood cholesterol nonprotein nitrogen, or uric acid Beatty (1952) studied experimental diabetes in rats but was unable to find any significant change in the blood ergothioneine levels in alloxan diabetes or after the administration of insulin or glucagon to pancreatectomized rats It was found, surprisingly, that blood ergothioneine levels fell after pancreatectomy The relation of ergothioneine to diabetes clearly needs further study

The effects of other pathological conditions on blood ergothioneine have been studied, but not thoroughly enough to establish positive effects Perhaps one exception to this statement is the relationship between tuber

mained that normal diets contained a factor necessary for the synthesis of ergothioneine Studies were therefore carried out on animals subsisting on stock diets It was first determined that the intestinal flora contributed no significant amounts of ergothioneine to the blood This was done by showing that Sulfasuxidine added to the diet of rats had no effect on the blood ergothioneine levels, and by showing that chickens raised in a germ free state exhibited normal blood levels (Melville and Horner, 1953) Then several possible precursors of ergothioneine, labeled with radioisotopes, were administered to animals and the blood or other tissues were examined for the presence of radioactive ergothioneine by chromatographic analysis on alumina columns (Melville *et al*, 1954) In this fashion, studies were car

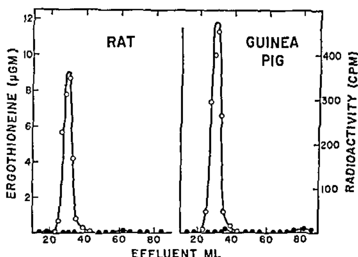


FIG 2 Distribution of ergothioneine (O) and radioactivity (●) in alumina chromatograms of red cells from rat and guinea pig which had received S^{35} methionine

ried out with histidine *2* C^{14}, thiolhistidine *2* C^{14}, methyl labeled C^{14} methionine, S^{35} methionine, C^{14} glycine, and C^{14} formaldehyde in the rat, with histidine *2* C^{14} in the chicken, with S^{35} methionine in the guinea pig, and with S^{35} cystine in the human (Melville *et al*, 1955a) Typical results from two of these experiments are shown in Fig 2 In no case was it possible to detect radioactivity in the ergothioneine from any of these animals

Although the characteristically high blood ergothioneine levels exhibited by the pig made it seem possible that this species did synthesize ergothioneine, *in distinction to the* rat, chicken, guinea pig, and human, a feeding experiment with S^{35} methionine and a young boar was undertaken by Melville *et al* (1955b) In contrast to the results of Heath *et al* (1953), no evidence for the presence of radioactivity in the *ergothioneine of the blood or* seminal vesicles could be found These conflicting results were resolved by

Heath *et al* (1957), who, on repetition of their earlier experiment with the boar, were unable to detect radioactivity in the isolated ergothioneine

It would seem fairly safe to generalize, from the negative results with the several species studied, that higher animals do not possess the ability to synthesize ergothioneine Since the ergothioneine of animal tissues is therefore dietary in origin, the compound must be present in several foodstuffs Tissue ergothioneine provides a dietary source for carnivorous animals, but the presence of the compound in the blood of herbivorous species suggests that it occurs in several plant materials

VII Plant Ergothioneine

The pronounced ability of diet to change the blood ergothioneine levels of animals has led several investigators to examine foodstuffs for ergothioneine The finding by Eagles and Vars (1928) that the addition of corn to the diet of pigs increased the amount of ergothioneine in the blood prompted them to examine corn for the presence of the compound From an acid hydrolyzate of zein they obtained a brownish red color with the Hunter diazo test which they ascribed to the possible presence of thiolhistidine, although they apparently did not test thiolhistidine itself Such colors with the diazo test are highly nonspecific, and Hunter (1951) has shown that corn contains at least two substances, unrelated to thiolimidazoles, which give red colors with the reagent These may well explain the results of Eagles and Vars Failure to detect either ergothioneine or thiolhistidine in plant material has been mentioned by Ashley and Harington (1930), Baldridge and Lewis (1953), and Melville and Horner (1953) Melville and Horner found that the usual methods of protein hydrolysis caused extensive destruction of added thiolhistidine or ergothioneine, which is further evidence that the hydrolyzates studied by Eagles and Vars did not contain these compounds

1 *Isolation*

Following the demonstration by Baldridge and Lewis (1953) of the marked ability of oats to serve as a dietary precursor of blood ergothioneine in rabbits, Melville and Eich (1956) undertook an investigation of this grain and were able to obtain chromatographic fractions which gave positive diazo tests for ergothioneine By the application of precipitation and chromatographic procedures to a 2 kilo sample of rolled oats (Table III), they were then able to isolate in crystalline form a substance which was identified as ergothioneine by several criteria, including infrared absorption

2 *Distribution*

The foregoing work, establishing the presence of ergothioneine in oats, permits the reasonable assumption that it is present also in other plants

No proof of this has yet been published However, it provides the most straightforward explanation for the ability of an aqueous acetone extract of corn to maintain blood ergothioneine levels (Melville *et al*, 1955b) From the feeding experiments of Potter and Franke (1935) it is obvious that the ergothioneine content of different cereal grains varies widely Their work suggests that this is also true of different samples of oats, and this is substantiated by data from the author's laboratory The variations in ergothioneine content of plants in different localities may well explain the geographical variations in blood levels noted by Hunter (1951) and others

TABLE III

ISOLATION OF ERGOTHIONEINE FROM OATS

Fraction	Weight	Ergothioneine content (*mg*)
Ground oats	2 kg	34
Ethanolic extract	65 gm	23
Lead acetate filtrate	34 gm	20
Alumina column—75% ethanol	857 mg	20
Alumina column—75% ethanol	283 mg	15
Cuprous oxide precipitation	82 mg	9 2
Alumina column—80% ethanol	8 6 mg	7 2
Crystallization	6 3 mg	6 3

3 Evidence for Nonsynthesis

Eich and Melville (paper in preparation for publication) have investigated the origin of plant ergothioneine In this work, sterilized oat seeds were allowed to grow in a sterile atmosphere and in contact with a nutrient medium containing high concentrations of S^{35} sulfate After 2 to 6 weeks the plants were extracted with an aqueous solution of nonisotopic ergothioneine and the extract was examined chromatographically for the presence of radioactive ergothioneine Several experiments of this kind were carried out, and in each case a low order of radioactivity, between 6 and 80 counts per minute (c p m), was found in the isolated ergothioneine Chemical tests indicated that this activity was not caused by the presence of a radioactive impurity

It is probable that the results of this experiment demonstrate the biosynthesis of ergothioneine However, it is highly questionable whether this synthesis can be ascribed to the plants Because of the high levels of radioactivity used (up to 2 mc in some experiments), even minute amounts of ergothioneine synthesized by contaminating microorganisms would be

detected (see Section VIII, below) Although mycostatic agents were added to the cultures and no gross contamination could be detected during or at the end of the experiment, it is impossible to rule out the presence of some viable cells which survived long enough to produce a trace of ergothioneine A control experiment in which the S^{35} sulfate was added at the end of the growth period yielded ergothioneine with no detectable radioactivity If the results are in fact due to the synthesis of ergothioneine by the plants, they demonstrate that the amounts synthesized during the early stages of growth are exceedingly small However, another factor points to nonsynthesis by oats, and that is the variability in ergothioneine content observed by Potter and Franke (1935), as determined by ability to increase blood ergothioneine levels In addition to this, Eich and Melville examined some samples of oat seeds which contained no detectable amounts of ergothioneine

4 *Incorporation of Exogenous Ergothioneine*

In line with the assumption that plant ergothioneine might be exogenous in origin, Eich and Melville studied the ability of plants to incorporate ergothioneine via the roots For this purpose, oat seedlings were suspended in solutions containing S^{35} ergothioneine After 6 hours the leaves were removed from the plants and an extract was made and subjected to chromatographic analysis Radioactive ergothioneine was found to be present in the leaves The isolated material showed 2900 c p m , compared to a total of 116,000 c p m in the ergothioneine originally added

This ready incorporation of ergothioneine by oat plants lends support to the possibility that plants synthesize little or no ergothioneine, but obtain it from the soil The discovery that many of the fungi commonly found in soil do synthesize ergothioneine (Melville *et al*, 1956) makes this idea particularly attractive Ergothioneine producing fungi are also present in the oat plants themselves, but it is unlikely that this source can alone account for the high levels encountered, for example in the oat samples from which ergothioneine was isolated

VIII Microbial Ergothioneine

1 *Molds*

It has long been believed that the ergothioneine of ergot is produced by synthesis in the causative fungus, *Claviceps purpurea* Confirmation of this belief was provided by Heath and Wildy (1956) and Ban and Stowell (1956) who obtained ergothioneine from *C purpurea* grown in chemically defined media Although ergothioneine was originally isolated from ergot (Tanret, 1909), it was not until recently that it was shown to be present in

other fungi In searching for the origin of plant ergothioneine, Melville *et al* (1956) obtained species of *Alternaria* and *Aspergillus* from oat seeds After the fungi were grown on chemically defined media and hot water extracts of the mycelia were chromatographed on alumina, positive diazo tests for ergothioneine were obtained Several other genera of fungi were

TABLE IV
ERGOTHIONEINE LEVELS IN FUNGI[a]

Organism	Ergo thioneine	Organism	Ergo thioneine
Alternaria tenuis	4	*Neurospora crassa*	85
Alternaria zinniae	2	*Neurospora tetrasperma*	27
Aspergillus carbonarius	18	*Penicillium notatum*	13
Aspergillus niger	12	*Pullularia pullulans*	34
Mucor mucedo	29		

[a] Values are expressed as milligrams per 100 gm. dry weight

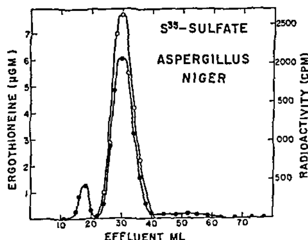


FIG 3 Distribution of ergothioneine (○) and radioactivity (●) in alumina chromatogram of aqueous extract of *Aspergillus niger* grown in the presence of S^{35} sulfate

then examined by the same technique, and in all cases evidence for the presence of ergothioneine was found (Table IV) To establish more firmly that ergothioneine was responsible for the positive diazo tests, two fungi, *Aspergillus niger* and *Neurospora crassa*, were grown in the presence of S^{35} sulfate and analyzed for the presence of radioactive ergothioneine In both cases, chromatographic analyses indicated that the diazo reacting substance was radioactive (e g Fig 3), and chemical tests showed that the

radioactivity was almost certainly attributable to ergothioneine As final proof, crystalline ergothioneine was isolated from a large scale culture of *Neurospora crassa* In addition to the fungi listed in Table IV we have also examined the large fleshy fungi, grown in their natural habitat, and found ergothioneine in concentrations of 10–100 mg per 100 gm of dried material in bracket fungi and in mushrooms from the United States, Chile, Italy, and China

2 Bacteria

Several bacteria have been examined for the ability to synthesize ergothioneine (Melville *et al*, 1956) The techniques were the same as those used for studying molds In sharp contrast, however, none of the bacteria which were grown in synthetic media gave any evidence of ergothioneine synthesis (Table V) This was confirmed in the case of *Staphylococcus*

TABLE V

BACTERIA SHOWING NO EVIDENCE OF ERGOTHIONEINE SYNTHESIS

Bacillus subtilis	*Pseudomonas fluorescens*
Clostridium perfringens	*Staphylococcus aureus* (*Micrococcus pyogenes* var *aureus*)
Corynebacterium xerose	*Streptococcus salivarius*
Escherichia coli	*Streptococcus pyogenes*
Lactobacillus casei	*Vibrio metchnikovii*
Mycobacterium spp	
Proteus vulgaris	

aureus (*Micrococcus pyogenes* var *aureus*) and *Escherichia coli* by growing the organisms in the presence of radioactive sulfur compounds In neither case did this more sensitive procedure reveal ergothioneine synthesis

It was found that bacterial cells are similar in one respect to mammalian cells with regard to ergothioneine, although they are not able to synthesize the compound they do possess the ability in varying degree to incorporate and concentrate exogenous ergothioneine This property is particularly evident with *S aureus* less marked with *Lactobacillus casei* or *Streptococcus faecalis*, and apparently lacking in *Pseudomonas fluorescens* Cultures of several bacteria, when grown in ordinary bacteriological media, may contain ergothioneine in the cells This has been traced to the presence of ergothioneine in some of the common components of bacteriological media beef extract, yeast extract and peptone The detection of ergothioneine in these materials was facilitated by utilizing the ability of *Staphylococcus aureus* to incorporate ergothioneine The bacterium was grown in a medium containing the material under study and then the cells were analyzed chromatographically (Melville *et al*, 1956)

3 Yeasts

The sharp distinction between the multicellular molds and the unicellular bacteria in ability to synthesize ergothioneine makes a study of yeasts of particular interest, since these latter organisms, which are considered to be primitive or degenerate forms of the molds, are frequently unicellular Unfortunately, very little information is yet available concerning their ability to synthesize ergothioneine D S Genghof and E Inamine in the author's laboratory obtained some interesting results in this direction *Saccharomyces cerevisiae* and *Torulopsis utilis*, when grown in chemically defined media, produced no detectable amounts of ergothioneine These results were confirmed with the aid of radioactive sulfate as a tracer Two other yeasts, *Saccharomyces carlsbergensis* and *Sporobolomyces salmonicolor*, likewise gave negative results by chromatographic analysis Several yeasts gave interfering colors in the diazo test, but it appeared that little or no ergothioneine was present Perhaps the most interesting results were obtained with *Geotrichum rugosum*, a member of a genus whose classification has been in doubt because it is less yeastlike and more moldlike in its properties, including the formation of a true mycelium, than most of the organisms which are classified as yeasts (Skinner *et al* , 1947) It was found that this organism produced high concentrations of ergothioneine, on the order of 40–110 mg per 100 gm of dry cells It is tempting to speculate that ergothioneine production may be of aid in taxonomic studies on yeasts and molds

There is clearly a need for further studies on the ergothioneine synthesizing abilities of microorganisms A study of the Actinomycetes, which are intermediate in nature between bacteria and true fungi, should yield interesting results Along this line, it should be noted that *Mycobacterium tuberculosis* like other bacteria, apparently does not produce ergothioneine, but does incorporate ergothioneine very efficiently Perhaps this is a reflection of its closer relationship than many other bacteria to the true fungi It is perhaps not too implausible to consider that there may be some connection between this property and the high ergothioneine levels noted in some cases of human tuberculosis, indeed it is possible that some strains of *M tuberculosis* do synthesize ergothioneine

Although studies of organisms such as the algae and slime molds must be carried out before the phylogenetic relationships of ergothioneine production can be adequately assessed, present data have determined that all four classes of the true fungi or Eumycetes possess this property Ascomycetes (*Claviceps*), Phycomycetes (*Mucor*), Basidiomycetes (mushrooms), and Fungi Imperfecti (*Penicillium*, etc) This synthesizing ability is lost or greatly attenuated in the yeastlike forms It is to be hoped that further studies may reveal a connection between ergothioneine and morphology,

reproduction, or spore formation and germination With regard to this last point, Heath and Wildy (1956) found ergothioneine only in the spores and not in the mycelium of *C purpurea* It is a common constituent of the mycelia of many other fungi, however (Melville *et al*, 1956) We have had occasion to examine the conidia of *Aspergillus niger* and *Neurospora crassa* and have found ergothioneine to be present

It seems likely that fungi are the primigenial source of most or all of the ergothioneine in man and other animals It may be ingested either directly in fungi such as mushrooms or those present in higher plants, or indirectly after assimilation by plants from the soil A rational explanation for the geographical variations in blood ergothioneine levels can be seen in the variations in the fungal complement of soils from different regions More speculative, perhaps is the thought that apparent decreases in blood ergothioneine levels observed in some localities during the last few decades may be related to changes in agricultural methods

IX Biosynthesis

The pathway of biosynthesis of ergothioneine has been studied in ergot (*Claviceps purpurea*) by Heath and co workers, and in *Neurospora crassa* by the author and his co workers The results of both of these independent lines of research are in agreement indicating a single biosynthetic route for ergothioneine in fungi

1 *Synthesis by Claviceps purpurea*

Heath and Wildy (1956) grew *C purpurea* in a chemically defined medium in the presence of acetate labeled in the methyl group with C^{14} After 10 days the fungus was collected, and the ergothioneine was extracted and degraded to trimethylamine and thiolurocanic acid by treatment with alkali The thiolurocanic acid was further degraded, by reduction with sodium amalgam and then oxidation with hydrogen peroxide, to imidazole propionic acid This in turn was esterified and degraded by fission of the imidazole ring with benzoyl chloride The resulting ester was hydrolyzed and then decarboxylated with copper chromite Radioactivity determinations on the various fractions permitted individual assay of the imidine carbon of the imidazole ring, the 5 carbon chain, the carboxyl group and the methyl groups of ergothioneine Histidine was isolated from a hydrolyzate of the fungal protein and was degraded to permit similar analyses Comparison of the two sets of data (Table VI) showed similarities in the distribution of radioactivity which suggested that ergothioneine was derived from histidine Histamine *2* C^{14} did not serve as a precursor (Wildy and Heath 1957) Further evidence that histidine is a precursor was obtained by growing the fungus in the presence of histidine *2* C^{14} and

histidine-α C^{14} (Heath and Wildy, 1958) The use of histidine-α C^{14} led to ergothioneine labeled only in the 5 carbon chain When histidine *2* C^{14} was the precursor, the ergothioneine was labeled almost solely in the C-2 atom of the imidazole ring, with a small amount of radioactivity in the methyl groups These results were considered to be proof that the intact imidazole ring is incorporated into the ergothioneine molecule This conclusion is open to argument, since a comparison of the specific activities of the histidine *2* C^{14} and the resulting C^{14} ergothioneine was not made However, the conclusion is fully substantiated by studies with *Neurospora* described below Wildy and Heath (1957) also showed that S^{35} methionine serves as a precursor for the sulfur atom of ergothioneine

TABLE VI

DISTRIBUTION OF RADIOACTIVITY IN ERGOTHIONEINE AND HISTIDINE SYNTHESIZED FROM ACETATE *2* C^{14} BY *Claviceps purpurea*

Moiety	Ergothioneine	Histidine	Ergothioneine / Histidine
Amidine carbon	2411	1368	1 76
5 Carbon chain	113	72	1 57
Carboxyl group	216	142	1 52
Methyl groups	500	—	—

[a] Values are expressed as counts per minute per milligram of carbon

2 Synthesis by Neurospora crassa

Studies on the ergothioneine synthesizing abilities of common molds have revealed that *Neurospora crassa* produces appreciable quantities of the compound (Table IV) This organism was therefore selected for studies in the author's laboratory of the pathway of ergothioneine biosynthesis The results have permitted a complete accounting for the origin of the ergothioneine molecule and have provided evidence for the time sequence of the steps involved in the biosynthesis

a Origin of the Imidazole Ring To study the precursor ability of histidine, *N crassa* was grown in the presence of histidine *2* C^{14} in a chemically defined medium for 24 hours (Melville *et al*, 1957) The mycelium was extracted with hot water, and the extract was chromatographed on alumina columns (Melville *et al*, 1954) The effluent fractions were analyzed for ergothioneine by the modified Hunter diazo test (Melville and Lubschez, 1953) and were also examined for radioactivity The results (Fig 4) showed a radioactive peak coinciding with the ergothioneine determinations Treatment of this material with strong alkali resulted in the formation of trimethylamine with negligible radioactivity, together with thiolurocanic acid which was highly

radioactive These results, demonstrating the precursor ability of histidine, are of greater significance when considered from the quantitative point of view, for it was found that when the C^{14} histidine concentration in the growth medium was made sufficiently high, the specific radioactivity of the thiolurocanic acid was identical with that of the histidine This indicates that the synthesis of ergothioneine from endogenous histidine was completely suppressed by the added C^{14} histidine, and that the intact imidazole ring of histidine is used in the biosynthesis, since if the ring were opened at the C-2 atom during the introduction of the sulfhydryl group it is probable that at least part of this labeled carbon atom would be lost

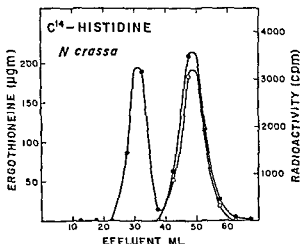


FIG 4 Distribution of ergothioneine (O) and radioactivity (●) in alumina chromatogram of aqueous extract of *Neurospora crassa* grown in the presence of histidine 2 C^{14}

b Origin of the Side Chain A reasonable assumption from the above data is that the whole histidine molecule is utilized in the biosynthesis of ergothioneine However, possible intermediates include urocanic acid, imidazolepyruvic acid, or sulfur containing derivatives of these compounds in which the α amino nitrogen atom of histidine has been removed To investigate this possibility histidine labeled with N^{15} was used as a precursor (Melville Ludwig, Inamine, and Rachele 1959) The N^{15} histidine was prepared biosynthetically by growing a large culture of *N crassa* in a medium containing N^{15} ammonium salts as the sole source of nitrogen, the labeled histidine was isolated from an acid hydrolyzate of the mycelial protein This N^{15} histidine which was uniformly labeled in all three nitrogen atoms was then used as a precursor of ergothioneine in a second culture of *N crassa* The isolated ergothioneine was degraded to thiolurocanic acid and trimethylamine Analyses of the histidine, ergothio

neine, and its degradation products (Table VII) demonstrated a high degree of retention of N^{15} in both the imidazole ring and the α nitrogen atom It can be concluded that the whole histidine molecule, *including the α nitrogen atom*, is incorporated into ergothioneine

c Origin of the Methyl Groups via Transmethylation When *N crassa* was grown in the presence of methyl labeled C^{14} methionine, the resulting ergothioneine was highly radioactive, and by degradation it was established that essentially all of the radioactivity was present in the methyl groups (Melville *et al*, 1957) Furthermore, neither free formate nor free formalde-

TABLE VII

DISTRIBUTION OF N^{15} IN ERGOTHIONEINE SYNTHESIZED FROM N^{15} HISTIDINE BY *Neurospora crassa*

Compound	N^{15} Content (atoms % excess)
Histidine (uniformly labeled)	4 83
Ergothioneine	3 78
Thiolurocanic acid	4 38
Trimethylamine	2 58

TABLE VIII

ISOTOPE CONTENT OF METHYL GROUPS OF ERGOTHIONEINE SYNTHESIZED FROM $C^{14}D_3$ METHIONINE BY *Neurospora crassa*

Compound	C^{14} (c p m /mmole methyl)	D (atoms % excess)	D C^{14}
Methionine	536 000	61 5	1 00
Trimethylamine	4 510	0 430	0 79

[a] The D C^{14} ratio of the methyl group of methionine is given an arbitrary value of unity

hyde is an intermediate in this process, since when either C^{14} formate or C^{14} formaldehyde was tested as a precursor, the resulting ergothioneine was labeled not only in the methyl groups but also in the thiolurocanic acid portion of the molecule These data suggest transmethylation as the reaction mechanism Proof of such a mechanism, however, demands the use of methyl groups in which the hydrogen atoms are labeled

Studies with methionine labeled in the methyl group with both C^{14} and deuterium have been carried out by Melville, Ludwig, Inamine, and Rachele (1959) Ergothioneine synthesized by *N crassa* from this doubly labeled methionine was degraded to trimethylamine, and the C^{14} and D content of this compound was compared with the isotope concentrations in the methionine methyl group The results (Table VIII) demonstrate an

appreciable retention of deuterium If the transfer of methyl carbon atoms from methionine to ergothioneine proceeded via formate or formaldehyde, then the ratio D C^{14} in the ergothioneine methyl groups could not be appreciably greater than one third in the case of formate, or two thirds in the case of formaldehyde, of the D C^{14} ratio of the methyl group of methionine Since the observed ratio is significantly greater than two thirds, at least one intact methyl group must have been transferred Unfortunately, the high dilution of isotopes in this experiment precluded the possibility of deriving information regarding the origin of the other two methyl groups

It was found that the dilution of C^{14} and D could be markedly reduced by using a methionineless mutant of *N crassa* Repetition of the experiment with this mutant, grown in the presence of doubly labeled methionine, yielded ergothioneine in which the trimethylammonium moiety was heavily labeled with both C^{14} and D (Table IX) Since the specific activities of the C^{14} and D in the trimethylamine are significantly greater than two thirds

TABLE IX

ISOTOPE CONTENT OF METHYL GROUPS OF ERGOTHIONEINE SYNTHESIZED FROM $C^{14}D_3$ METHIONINE BY METHIONINELESS *Neurospora crassa*

Compound	C^{14} (c p m /mmole methyl)	D (atoms % excess)	D C^{14}
Methionine	90 300	77 2	1 00
Trimethylamine	72 500	61 4	0 99

[a] The D C^{14} ratio of the methyl group of methionine is given an arbitrary value of unity

of the specific activities of these isotopes in the methyl group of methionine, all three methyl groups of the ergothioneine must be labeled with isotope This fact, together with the fact that the D C^{14} ratio in the trimethylamine is identical with the D C^{14} ratio in the methionine can be considered as conclusive proof that all three methyl groups have been transferred from methionine by transmethylation reactions This result is of special significance in that it is apparently the first demonstration that the trimethylammonium radical of a betaine can be synthesized in nature by means of a triple transmethylation process

d Origin of the Sulfur Since *N crassa* grows in media containing inorganic sulfate as the sole source of sulfur, it is obvious that the sulfur of ergothioneine must be derived ultimately from sulfate, and this is readily confirmed by the use of S^{35} sulfate (Melville *et al* , 1956) To determine whether any known sulfur compounds are more immediate precursors, competition experiments were carried out in which *Neurospora* cultures were grown in the presence of S^{35} sulfate and various sulfur containing compounds (Melville *et al* , 1957) The results of these experiments (Table

X) *demonstrate that* thiosulfate and the sulfur containing amino acids are efficient precursors Thiosulfate undoubtedly owes its effectiveness to its ready conversion to cysteine, inasmuch as this conversion has been shown to occur in *Aspergillus nidulans* (Shepherd, 1956) The precursor ability of cystine and methionine was confirmed by the use of the S^{35} labeled amino acids The most effective precursor, cysteine, is probably the immediate precursor of the sulfur atom of ergothioneine

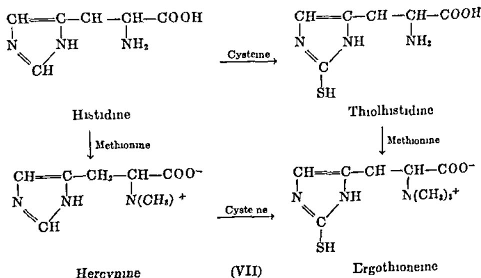
e Sequence of Biosynthetic Reactions The preceding data account for the origin of the ergothioneine molecule in the three amino acids histidine,

TABLE X

SUPPRESSION OF S^{35} SULFATE INCORPORATION INTO *Neurospora* ERGOTHIONEINE

Competitor	Ergothioneine (c p m /mg)	Suppression (%)
None	23,000	
Thiocyanate	23,800	0
Thioacetamide	20,200	12
Choline sulfate	17,300	25
Thiosulfate	3,400	85
Methionine	3,600	84
Cystine	2,000	91
Cysteine	290	99

methionine, and cysteine The time sequence of the reactions remained to be determined Although several synthetic routes are possible, the two most straightforward pathways (VII) involve either thiolhistidine or hercynine, the betaine of histidine, as intermediates



(VII)

When *N crassa* was grown in the presence of thiolhistidine *2* C^{14}, the ergothioneine which was produced contained a negligible amount of radio activity (Melville *et al*, 1957) The possibility that this negative result might be due to impermeability of the cell membrane to thiolhistidine is unlikely since appreciable amounts of radioactivity were present in extracts of the mycelium It can be concluded that thiolhistidine is not an inter mediate in the biosynthesis of ergothioneine

The possibility that hercynine is an intermediate in the biosynthesis has been studied by Askari and Melville (in preparation for publication) This betaine has been known for many years, it was found in mushrooms (Kutscher, 1911, Reuter, 1912 Winterstein and Reuter, 1913) and has been obtained as an oxidation product of ergothioneine (Barger and Ewins, 1911 1913) Its synthesis from α chloroimidazolepropionic acid and tri methylamine has been described (Engeland and Kutscher, 1912, Pasini and Vercellone, 1955) However, it has been well characterized only as the picrate and gold salt Studies of its relationship to ergothioneine were greatly facilitated by the preparation of the free base in crystalline form The optically active compound was prepared from ergothioneine by oxida tion with ferric chloride as described by Barger and Ewins (1911) and was crystallized from a methanol ether mixture

A competition experiment was carried out to determine whether the presence of added hercynine would decrease the incorporation of histidine *2* C^{14} into ergothioneine by *N crassa* No effect could be found It was possible that this was due to impermeability of the cell wall to hercynine Since W Feldman in our laboratory had found that *N crassa* in growing cultures is relatively impermeable to ergothioneine, but becomes more permeable when it is starved, the same technique was tried with hercynine When the competition experiment was carried out with mycelium sus pended in water rather than growth medium a definite effect was ob served, with a marked decrease in the uptake of histidine *2* C^{14} in the pres ence of hercynine (Fig 5)

As final proof that hercynine is an intermediate, radioactive hercynine labeled in both the imidazole ring and the methyl groups with C^{14} was used as a precursor The labeled compound was prepared from C^{14} ergothioneine which in turn was prepared biosynthetically by growing *N crassa* in the presence of C^{14} formate When the doubly labeled hercynine was added to starving cells of *N crassa* the ergothioneine produced was radioactive The isolated ergothioneine and the C^{14} hercynine were both degraded with alkali to determine the distribution of C^{14} in the molecules The ratio of imidazole to methyl C^{14} content of the hercynine was 1 07, while the ergo thioneine synthesized from it showed a ratio of 1 04 This can be considered as proof that the intact hercynine molecule is incorporated into ergo thioneine

f Presence of Hercynine in Neurospora It had been noticed that when *N crassa* was grown in the presence of methyl labeled C^{14}-methionine, C^{14} histidine, or C^{14} formate, a radioactive substance preceded ergothioneine in alumina chromatograms of the mycelial extracts (Figs 4 and 5) Fractions containing this substance gave yellow to orange colors in the diazo test, which are typical of the colors given by hercynine Authentic hercynine is eluted at the same place on the chromatogram as the radioactive substance, and both show the same behavior in paper chromatography Crystallization of carrier hercynine in the presence of the radioactive substance yielded crystalline radioactive hercynine which on decomposition with alkali gave radioactive urocanic acid It is probable, therefore, that hercynine is a normal constituent of *Neurospora* The increase in amount of radioactive hercynine evident in the competition experiment (Fig 5) is understandable if the small amount of C^{14} hercynine being continuously formed from C^{14} histidine is diluted and "trapped" by the added nonisotopic hercynine before it can be converted to C^{14} ergothioneine

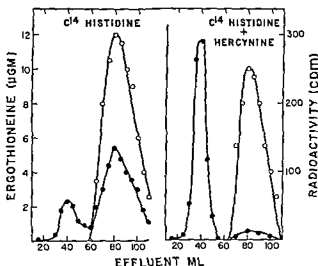


FIG 5 Distribution of ergothioneine (O) and radioactivity (●) in alumina chromatograms of extracts of *Neurospora crassa* showing effect of nonisotopic hercynine on the incorporation of histidine *2* C^{14} into ergothioneine

From the foregoing results it can be assumed that the occurrence of hercynine in fungi is explicable on the basis of its formation as an intermediate in the synthesis of ergothioneine Whether it is also a catabolic product of ergothioneine metabolism is not known In fact, very little is known about the catabolism of ergothioneine Neufeld *et al* (1958) have shown that it is oxidized at the sulfur atom by an enzyme from the fungi *Piricularia oryzae* and *Polyporus versicolor* The product of the reaction was not determined, but if the oxidation is similar to that effected by chemical

agents, hercynine might well be formed Another pathway of decomposition has been shown by Yanasugondha and Appleman (1957), who found that *Alcaligenes faecalis* degraded ergothioneine to trimethylamine and thiol urocanic acid That ergothioneine can be metabolized in animal tissues is suggested by the formation of inorganic sulfate from S^{35} ergothioneine in the rat (Heath, 1953)

Ackermann and his associates have obtained evidence for the presence of hercynine as well as ergothioneine in the king crab (1958) and in the red cells of cattle and the seminal fluid of the boar (1959) They suggest that hercynine may be a precursor for the biosynthesis of ergothioneine in these species However, the inability to detect ergothioneine biosynthesis in the boar (Melville *et al*, 1955b, Heath *et al*, 1957) makes it seem more likely that the hercynine of animal tissues is related to ergothioneine only as a degradation product

X. Biological Activity

Numerous attempts to define a biological activity for ergothioneine have been recorded in the literature Many of these have given negative results, but a few have yielded interesting positive effects The biological effects ascribed to ergothioneine have not yet been adequately assessed with regard to their physiological significance, and it is likely that the future will reveal functions which are at present unrecognized It is to be hoped that the varied activities of apparently unrelated nature which have been described for ergothioneine will eventually become parts of a unified concept of action

1 *Pharmacology*

Tainter (1927) found no effect of injected ergothioneine on respiration, pulse rate pupil diameter, or blood sugar level in the rabbit or on blood pressure, pulse rate respiration, or biliary secretion in the anesthetized cat The substance had no effect on excised smooth muscle Similarly, Trabucchi (1936) observed no effect on uterine contraction, although an increase in the contraction rate of the isolated frog heart, with diastolic arrest at high concentrations, was found Trabucchi also noticed an antagonistic action of injected ergothioneine toward some of the symptoms of beriberi in pigeons, and a detoxifying effect toward cyanide poisoning in rats These activities have apparently not been investigated further

2 *Nutrition*

It was early discovered that ergothioneine could not replace, even partially the dietary histidine requirement of the growing rat (Eagles and Cox, 1928) Similar negative results have been obtained with thiolhistidine

(Neuberger and Webster, 1946) In the author's laboratory several groups of rats have been maintained on purified diets for periods up to four months from the time of weaning, with the inclusion of ergothioneine at a level of 0 005% This level of dietary ergothioneine leads to blood levels in the normal range for the rat Comparison of the ergothioneine fed animals with ergothioneine depleted control animals revealed no significant differences in growth rate, food consumption, or gross appearance The same results were obtained whether the nitrogen source in the diet was casein, fortified zein, or a mixture of purified amino acids

An experiment to determine the effect of ergothioneine on the growth of neoplastic tissue was carried out by A Askari and the author in collaboration with D A Clarke of the Sloan Kettering Institute Fifteen weanling mice were maintained on a purified, casein containing diet for 85 days, and another group of fifteen was maintained on the same diet supplemented with 0 005% ergothioneine Nine animals from each group were then implanted with sarcoma 180 No differences in the growth rates of the tumors in the ergothioneine depleted and ergothioneine fed animals were discernible No ergothioneine was detectable in the blood or liver of the control group, or in the tumors of these animals Apparently tumor tissue, like normal mammalian tissue, is not capable of synthesizing ergothioneine It is of interest, however, that the tumor tissue from the ergothioneine fed animals contained high levels of ergothioneine Six animals, analyzed 8 or 16 days after tumor implantation, showed an average ergothioneine level in the tumors of 6 7 mg per 100 gm of tissue, compared to levels of 3 4 in the blood and 6 6 in the liver Whether an affinity for ergothioneine is characteristic of other types of neoplastic tissues is not known

Efforts to identify ergothioneine as a growth factor for lower organisms have so far not been successful Hogg and Elliot (1951) found that it could not replace the histidine requirement of *Tetrahymena geleii*, nor did it stimulate the lag phase of growth of *Staphylococcus albus* (Sloane and McKee 1952) Work in the author's laboratory has failed to uncover any effect of ergothioneine on the growth of various bacteria

In contrast to the experiments with whole animals, an interesting effect of ergothioneine on the metabolism of isolated tissues has been uncovered by Morton and Morgan (1958) Embryonic chick heart fibroblasts when grown in a synthetic medium required cystine and D or L methionine for maximum survival time The decreased survival time seen in a cystine free medium was increased significantly by the addition of ergothioneine at a concentration of 0 1 μg per milliliter Higher concentrations showed a toxic effect The presence of L-methionine was necessary for the increased survival time effected by ergothioneine, when D methionine was used, or when no methionine was present, only a toxic effect of ergothioneine could

be seen Lanthionine and oxidized glutathione, but not reduced glutathione, likewise increased the survival time in cystine free media, but with these compounds either D or L-methionine permitted the effect No explanation of these results is apparent at present

In view of the known lipotropic effect of betaine, the activity of ergothioneine as a lipotropic agent is of interest No work has been published, but Best and Ridout (1939) have mentioned that negative results were obtained in unpublished experiments

3 *Protective Effects*

The protection against radiation injury shown by some sulfhydryl and thiourea derivatives has led to the testing of ergothioneine for such properties, particularly since it is a normal constituent of some radiation sensitive tissues and can be readily increased in amount in these tissues Alexander *et al* (1955) found that ergothioneine, injected into mice a few minutes before irradiation with a lethal dose of X rays afforded no protection In the author's laboratory, eight adult rats which had been depleted of ergothioneine were each given a total of 20 mg of ergothioneine in four intraperitoneal injections over a period of 6 days prior to irradiation with 800 r of X rays No difference in survival time was seen between these animals and a control group which had received no ergothioneine Thiol histidine is likewise inactive as a protective agent (Langendorff *et al*, 1956)

St Garay (1956) found that the inhibition of germination of ergot conidia by hydrogen peroxide was overcome by the presence of an equivalent amount of ergothioneine This is hardly surprising inasmuch as ergothioneine readily reduces hydrogen peroxide St Garay found ergothioneine, not in the conidia, but in the ' honeydew ' secretion which accompanies the formation of conidia It was suggested that both ergothioneine and catalase, which is also present in honeydew, protect the fungus against the toxic effects of hydrogen peroxide A similar protective effect of ergothioneine against inhibition of fructolysis in semen by peroxide had been shown earlier by Mann and Leone (1953) It is interesting however, that Tosic and Walton (1950) have shown that boar semen, which is richest in ergothioneine is least able to decompose peroxide when compared with the semen of the bull, rabbit stallion, or ram Mann and Leone also showed that ergothioneine protects against the inhibiting effects of sulfhydryl binding agents on sperm motility, seminal fructolysis, muscle glycolysis, and yeast fermentation

An implication of a different kind of effect on seminal metabolism is made by Haag and MacLeod (1959), who found that an ergothioneine containing extract of human semen caused a marked depression of sperm motility and glycolysis However, pure ergothioneine did not produce this

(Neuberger and Webster, 1946) In the author's laboratory several groups of rats have been maintained on purified diets for periods up to four months from the time of weaning, with the inclusion of ergothioneine at a level of 0 005% This level of dietary ergothioneine leads to blood levels in the normal range for the rat Comparison of the ergothioneine fed animals with ergothioneine depleted control animals revealed no significant differences in growth rate, food consumption, or gross appearance The same results were obtained whether the nitrogen source in the diet was casein, fortified zein, or a mixture of purified amino acids

An experiment to determine the effect of ergothioneine on the growth of neoplastic tissue was carried out by A Askari and the author in collaboration with D A Clarke of the Sloan-Kettering Institute Fifteen weanling mice were maintained on a purified, casein containing diet for 85 days, and another group of fifteen was maintained on the same diet supplemented with 0 005% ergothioneine Nine animals from each group were then implanted with sarcoma 180 No differences in the growth rates of the tumors in the ergothioneine depleted and ergothioneine fed animals were discernible No ergothioneine was detectable in the blood or liver of the control group, or in the tumors of these animals Apparently tumor tissue, like normal mammalian tissue, is not capable of synthesizing ergothioneine It is of interest, however, that the tumor tissue from the ergothioneine fed animals contained high levels of ergothioneine Six animals, analyzed 8 or 16 days after tumor implantation, showed an average ergothioneine level in the tumors of 6 7 mg per 100 gm of tissue, compared to levels of 3 4 in the blood and 6 6 in the liver Whether an affinity for ergothioneine is characteristic of other types of neoplastic tissues is not known

Efforts to identify ergothioneine as a growth factor for lower organisms have so far not been successful Hogg and Elliot (1951) found that it could not replace the histidine requirement of *Tetrahymena geleii*, nor did it stimulate the lag phase of growth of *Staphylococcus albus* (Sloane and McKee, 1952) Work in the author's laboratory has failed to uncover any effect of ergothioneine on the growth of various bacteria

In contrast to the experiments with whole animals, an interesting effect of ergothioneine on the metabolism of isolated tissues has been uncovered by Morton and Morgan (1958) Embryonic chick heart fibroblasts when grown in a synthetic medium required cystine and D or L-methionine for maximum survival time The decreased survival time seen in a cystine free medium was increased significantly by the addition of ergothioneine at a concentration of 0 1 μg per milliliter Higher concentrations showed a toxic effect The presence of L-methionine was necessary for the increased survival time effected by ergothioneine, when D methionine was used, or when no methionine was present, only a toxic effect of ergothioneine could

be seen Lanthionine and oxidized glutathione, but not reduced glutathione, likewise increased the survival time in cystine free media, but with these compounds either D or L methionine permitted the effect No explanation of these results is apparent at present

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effect It is evident that the relation of ergothioneine to sperm metabolism needs further clarification

Ergothioneine is one of several substances which show a protective effect against the degradation of insulin by beef liver enzymes (Williams and Berg, 1956) It is of interest in this connection that the ability of sulfhydryl compounds such as cysteine and glutathione to inactivate insulin *in vitro* is not exhibited by ergothioneine (Schock *et al*, 1935)

The work of Spicer *et al* (1951) suggests the interesting possibility that ergothioneine may have a physiological role in maintaining hemoglobin in the ferrous form These workers found that the red blood cells from rabbits which had been fed purified diets showed an abnormally rapid formation of methemoglobin after treatment of the cells with nitrite The red cells from ergothioneine fed animals showed slower rates of nitrite-induced methemoglobin formation A similar effect has been observed with rats (Mortensen, 1953) Whether the effect is mediated through the enzyme system which normally catalyzes the conversion of methemoglobin to hemoglobin, or whether it is the result of a nonenzymatic protective effect of ergothioneine is not known The picture is complicated by the fact that methemoglobin is not only produced at a faster rate in ergothioneine depleted cells, but it also disappears at a faster rate *In vitro* studies with red cell peroxidase have failed to show any effect of ergothioneine on the oxidation of hemoglobin (Mills, 1959)

4 Effects on Thyroid Function

The potent inhibitory effect of thiourea derivatives on thyroid function suggests the possibility that ergothioneine may act as a regulator of thyroid activity Indeed, the first experiments in this direction suggested that this was so, for Lawson and Rimington (1947) observed an antithyroid activity for ergothioneine, comparable to that of thiouracil, after the injection of ergothioneine into rats at a dosage level of 2 mg per kilogram Activity was measured by the decrease in iodine content of the thyroid However, Astwood and Stanley (1947) could find no effect on iodine content or weight of the thyroid after injecting much larger amounts of ergothioneine into rats Inability to obtain an effect of ergothioneine on I^{131} uptake, or on size, weight, or histology of the thyroid gland has been noted by Wilson and McGinty (1949), Searle *et al* (1950), Heath *et al* (1952), Baldridge (1955), and Mackenzie and Mackenzie (1957) No adequate explanation of the original positive results is yet at hand The inactivity of ergothioneine is in sharp contrast to the marked activity of thiolimidazole and its 4 alkyl derivatives (Searle *et al*, 1950) However, a strong inhibitory effect of ergothioneine on the *in vitro* conversion of acetyldiiodotyrosine to acetyl thyroxine has been observed by Pitt Rivers (1948)

5 *Effects on Isolated Enzyme Systems*

There are several instances in which ergothioneine has been noted to be inactive in enzymatic reactions These include the inability of ergothioneine to replace glutathione as a cofactor for yeast glyoxylase (Woodward, 1935) or formaldehyde dehydrogenase (Strittmatter and Ball, 1955) or to replace betaine or dimethylthetin as a methyl donor for methionine synthesis by a betaine transmethylase system (Sloane *et al*, 1955) It has been mentioned as being inactive as an acceptor in acyl transfer reactions mediated by imidazole transacetylase (Stadtman, 1954) A positive effect of ergothioneine has been described by Leone (1955) who found that the activity of uricase was increased by ergothioneine or Versene He ascribes this effect to the chelation of ergothioneine or Versene with trace amounts of inhibitory metals present in the enzyme preparation

A finding which has elicited considerable interest is that of Grossman and Kaplan (1958) indicating that ergothioneine is responsible for the sensitivity of certain ribosidases to inhibition by nicotinamide This was discovered during attempts to purify nicotinamide riboside phosphorylase, an enzyme present in human red blood cells which catalyzes a phosphorolytic splitting of nicotinamide riboside to nicotinamide and ribose phosphate The enzyme preparation, which was initially inhibited by the presence of nicotinamide, became nicotinamide insensitive after ammonium sulfate fractionation An extract of red cells, when added to the enzyme, caused the enzyme to regain sensitivity to inhibition by nicotinamide The substance in the red cell extract behaved chemically and chromatographically like ergothioneine, and indeed ergothioneine was found to be capable of making the enzyme sensitive to inhibition by nicotinamide Furthermore both the red cell extract and ergothioneine were capable of imparting nicotinamide sensitivity to a DPNase" (diphosphopyridine nucleotidase) preparation from *Neurospora crassa* This enzyme, which is normally nicotinamide insensitive, catalyzes the hydrolytic fission of the nicotinamide ribosyl linkage in DPN The inhibition of the enzymatic reaction by nicotinamide when ergothioneine is present is of the noncompetitive type, and is accompanied by an increased rate of exchange between free nicotinamide and the nicotinamide moiety of the DPN molecule, as was shown by the use of C^{14} nicotinamide

The mechanism of the ergothioneine effect was suggested as involving the formation of a ribosyl linkage with the ergothioneine molecule, thereby retaining the bond energy of this linkage and permitting an exchange reaction with nicotinamide to occur The authors suggest the imidazole nitrogen atom or the quaternary nitrogen atom of ergothioneine as the point of formation of a ribosyl bond It seems to this reviewer that the thiol thione tautomerism of the thiolimidazole system makes the sulfur

atom a likely point of formation of a thio riboside linkage with sufficient bond energy to allow subsequent ribosyl exchange However, Grossman and Kaplan indicate that N[1] methylnicotinamide is produced during the cleavage of nicotinamide riboside, which suggests involvement of the quaternary nitrogen atom of ergothioneine Although Grossman and Kaplan have designated ergothioneine as a coenzyme of red cell nicotinamide riboside phosphorylase, this would seem to be an optimistic term since it has not been shown that ergothioneine acts catalytically

The physiological significance of this curious effect is at present unknown It remains to be determined whether ergothioneine is responsible for the nicotinamide sensitivity of the DPNases of red cells, spleen, and brain It may be noted that rat brain contains little or no ergothioneine The in sensitivity of *Neurospora* DPNase toward inhibition by nicotinamide can be explained by the fact that the procedure used for purification of the enzyme would also remove ergothioneine W Feldman in the author's laboratory has found that all of the determinable ergothioneine of *N crassa* is apparently present in the mycelial cytoplasm in an unbound form

One possible method of assessing the significance of the ergothioneine effect arises from the work of Kaplan *et al* (1954), who have suggested that the toxicity of 3 acetylpyridine in animals is due to an exchange of the drug with the nicotinamide moiety of DPN in the tissues If this exchange reaction is facilitated by ergothioneine, it would be expected that an ergo thioneine depleted animal would show increased resistance to the toxic effects of 3 acetylpyridine Experiments in the author's laboratory with ergothioneine fed and ergothioneine deficient mice have demonstrated no differences in the response to the toxic effects of the drug In other experi ments with rats, it has been observed that the DPN levels in the liver are not significantly affected by the presence or absence of ergothioneine

It is obvious from the foregoing review that much remains to be done to confirm, explain, or assess the significance of the various biological activities which have been ascribed to ergothioneine The suggestion sometimes voiced that ergothioneine is an accidental component of animal cells, with no specific function, cannot of course be effectively refuted until a function of recognized significance is uncovered The ability to produce ergothioneine deficiencies in animals without the appearance of gross deficiency symptoms comes as no surprise, since otherwise its essentiality would undoubtedly have been discovered years ago incidental to the many studies which have been carried out with animals maintained on purified diets It should be pointed out, however, that the methods available for ergothioneine detec tion are relatively insensitive, and the existence of trace amounts of free ergothioneine, or larger amounts of bound ergothioneine, is possible in the presumably ergothioneine free animal The long term effects of ergo

thioneine depletion have not been adequately studied However, it does seem likely on the basis of presently available evidence that ergothioneine is not an essential constituent of animal cells On the other hand, the avidity with which dietary ergothioneine is incorporated into the tissues of all animals, the tenacity with which it is held there, and its characteristic, nonuniform pattern of distribution in these tissues are all facts which hint that there is a purpose in its presence Perhaps ergothioneine is concerned with an alternative pathway of metabolism Perhaps its function becomes apparent only under conditions of stress Perhaps variations in ergo thioneine levels may help to explain the differences in metabolic patterns exhibited by individual members of a species The answers to questions such as these must come from future research

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Mechanisms of Action of Estrogens[1]

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I INTRODUCTION

Since the isolation some thirty years ago of steroid compounds which in minute doses produced the estrous response (Doisy *et al*, 1929, 1930a 1930b, Butenandt, 1929 Butenandt and von Ziegner 1930, Dingemanse *et al* 1930, Marrian 1930), great interest has been directed toward an explanation for these profound biological effects The qualitative and quantitative descriptions of the anatomic changes, both gross and microscopic induced by the estrogens constituted the first attempts to define their action in physiological terms However, until the framework of our knowledge of intermediary metabolism had been erected this approach was limited Our present, albeit imperfect understanding of energy production and utilization of the synthesis, degradation and translocation of tissue constituents and the localization of these functions within the cell provides the matrix within which hormones such as estrogens can exert their specific and controlling effects upon metabolic processes Included in this picture of the broad outlines of intermediary metabolism are the reactions in

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volved in the synthesis and catabolism of the estrogens, thereby establ ing a link between the main pathways of metabolic processes and stances which direct and control these processes This unfolding of knowledge of intermediary metabolism has been accompanied by the velopment of methods sufficiently refined to permit the beginnings of examination of estrogen action at the molecular level The physiolog effects of estrogens in the whole animal have been the subject of numer qualitative and quantitative descriptions The anatomical changes duced in the uterus of the immature or ovariectomized rodent (Bulbring Burn, 1935, Lawson *et al*, 1939, Evans *et al*, 1941), and the respons the chick oviduct to the administration of minute doses of estrogen (He 1948), are well recognized and easily discernible changes In addition these gross alterations, there are changes in the cellular population of vaginal epithelium (Stockard and Papanicolaou, 1917, Allen and Do 1923), changes in the mammary gland, and other more subtle effects estrogenic hormones which include those upon electrolyte metabol (Thorn and Engel, 1938) and upon the functional state of the ante lobe of the pituitary gland or of the hypothalamus These latter chan cannot yet be described entirely satisfactorily in terms of changes in m phology These effects upon the target tissues are the phenomena wh must be explained in terms of biochemical or biophysical events under specific control of the estrogenic hormones

At the biochemical level there is a small but growing body of informat concerning effects of estrogenic hormones on biochemical processes A complete concept of mechanism of action of estrogens must ultimat bridge the gap between these phenomena and the changes noted abo It is clear from the outset that at the present time no such bridge can built Experimental attack from both directions should serve to narrow t gap and ultimately establish a logical connection between the events the molecular level and those occurring in the whole animal which a seen as morphologic changes in the target tissue Our knowledge of mech nisms of hormone action is certainly less well developed than our und standing of the biochemical functions of certain vitamins However, it no more possible to explain the functional and anatomical changes induc by estrogen administration to the susceptible animal in terms of the pre ently known effects of estrogenic hormones on biochemical systems th it is possible to explain the syndrome of pellagra in terms of defects in t functioning of the known biochemical systems in which niacinamide pla a part

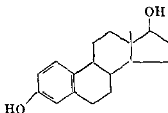
II Structure and Biological Activity

The problem of understanding the mechanisms of action of estrogens a difficult one for several major reasons First, estrogenic activity is ass

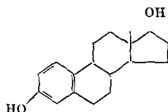
ciated with a wide diversity of chemical structures, secondly, estrogens, like other steroid hormones, are not required for life These two points and several of their consequences are worthy of more detailed discussion

Although estradiol 17β and/or estrone are believed to be the primary secretory products of the ovary in the human, the sow, and certain other large animals much experimental work has been carried out in the mouse and in the rat, in these species it is not known whether the same compounds are the primary secretory products although mouse and rat tissues respond to estradiol and estrone Furthermore, the nature of the endogenous substance which acts upon the target tissue is not known It is believed that the primary secretory product of the ovary is free estradiol or estrone, but in the blood stream the estrogens appear to be transported largely in conjugated form (Purdy *et al* 1959) Whether or not these conjugates are cleaved in the target tissue prior to exerting their hormonal action is not known

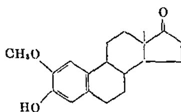
Estrogenic activity is a property of many types of natural and synthetic compounds First and foremost are the naturally occurring steroidal estrogens and the related compounds prepared in the laboratory (I II III),



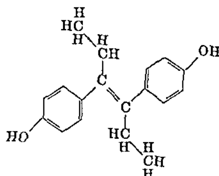
(I) Estradiol 17β



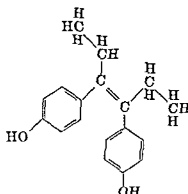
(II) Estradiol 17α



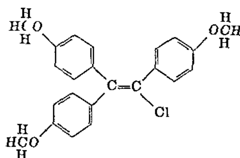
(III) 2 Methoxyestrone



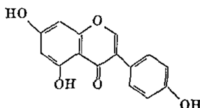
(IV) *trans* Diethylstilbestrol



(V) *cis* Diethylstilbestrol



(VI) Trianisyl chloroethylene



(VII) Genistein

but there are other substances such as stilbene derivatives (IV, V), the halogenated triphenylethylene (VI), and compounds of the type of genistein (VII), a plant estrogen, which share these biological properties Synthetic, nonsteroidal estrogens are reviewed by Solmssen (1945) and Grundy (1957),

and correlations between structure and biological activity are discussed by Dodds (1957) Plant estrogens are reviewed by Bradbury and White (1954) The only common structural feature of these compounds is the aromatic nucleus What is remarkable, however, is that within each group of compounds which can elicit the estrous response, there is a high degree of structural and steric specificity, very small changes in structure suffice to obliterate or markedly diminish the biological activity Thus, for example, estradiol 17β (I) is a highly potent compound in most of the test systems but its epimer, estradiol 17α (II), is essentially inactive Furthermore, the addition of substituents to a molecule such as estrone which possesses a high level of estrogenic activity may diminish the activity several thousandfold, e g 2 methoxyestrone (III) (Kraychy and Gallagher, 1957) Similarly, *trans* diethylstilbestrol (IV) is many times more active than is its *cis* isomer (V) (Dodds, 1957) Any mechanism proposed to explain the action of these substances must take into account not only the high degree of chemical and stereospecificity within a group of estrogenic substances having a common structural pattern, but also the possession of this activity by many compounds of widely differing structural types Theoretically, this difficulty may be circumvented in two ways First, by postulating that these substances are precursors of the true hormone and that in the body they are all converted to the same or to similar biologically active materials This is a difficult position to maintain in view of the diversity of chemical types and the fact that some of the synthetic estrogens are active in doses of the same order of magnitude as the effective doses for the most active naturally occurring compounds However, it should be recalled that there are instances in which an administered inactive compound is transformed in the body to an active compound (Emmens, 1941) Second it may be postulated that there are multiple receptors for the many structures which are associated with estrogenic activity and that interaction of the estrogen with any of them suffices to set in motion the processes leading to the measured response Thus for example estradiol, diethylstilbestrol and genistein need not necessarily affect the same systems at the molecular level, although the effects upon the whole animal are indistinguishable

This sharing of a biological activity by chemical compounds of many structural types is somewhat reminiscent of the situation with carcinogens Agents as different as ionizing radiation inorganic ions polycyclic aromatic hydrocarbons, and azo dyes have the common property of setting in motion a chain of biochemical events which leads to the development of a tumor

Structural specificity of estrogenic substances has, in addition, several important biological components The first is the situation in which com

pounds are highly potent estrogens when tested in one species and tive in another Substances of the doisynolic acid series are effective and parenteral estrogens in the rat and yet are inactive in the n (MacCorquodale *et al* 1934, Miescher, 1944, Tschopp, 1946) A se biological component is typified by the conditioned response of the oviduct to estrogens (Hertz, 1948) A full response is elicited only the bird is maintained on an adequate dietary level of folic acid In folic acid deficient birds, as shown by Hertz, the full growth of the ov cannot be achieved, regardless of the dose of estrogen administered

The second general point made above was that estrogens, like steroid hormones, are not essential for life This is implicit in the that the gonadectomized, adrenalectomized animal can be maintaine sodium chloride replacement therapy This suggests either that the e gens are dispensable and that the processes which they regulate can op at a basal level in their absence or that there are other endogenous or e enous sources of estrogen which suffice to meet the basic requirem Although it is not possible to exclude this eventuality completely, a i satisfactory working hypothesis is that the estrogens are not essentia life

III Mechanisms

In considering the types of mechanism by which estrogens may e their activities, several general approaches have been made The con that the action of estrogens and other hormones requires the intact has its protagonists, and in this view the function of the hormone is to fluence permeability of the cell membrane and thus exercise control the passage of metabolically important substances both inorganic or org into and out of the cell By thus altering concentrations of reactants wi the cell, metabolic processes can be controlled As a subsidiary to hypothesis, it has been postulated that even within the cell the m branous envelopes of such subcellular particles as the mitochondria m also constitute sites of action of hormones by the same general typ mechanism The permeability hypothesis in its various ramifications been discussed by Hechter (1955) in a recent review article There ar the present time few direct experimental data bearing upon the effec estrogens at physiological concentrations in influencing cell permeability

Considerable attention has been given to the concept that hormo exert their effects by acting in some way upon enzyme systems In gene two principal modes of action have been considered In the first v thought that the hormones function as activators or inhibitors of enzym reactions, and in the second view, the hormones are considered as act participants in enzymatic reactions In the first case, the hypothesis i

restatement of the experimental finding, since no more detailed mechanism susceptible of experimental investigation has yet been proposed The second concept, namely, that the hormones function as participants in enzymatic reactions derives in part from our concepts of the functions of those vitamins which are known to act as coenzymes Further appeal is given to this hypothesis by the fact that many of the steroid hormones are subjected to oxidative and reductive reactions during the course of their transformations to the biologically inactive excretory products It is indeed, attractive to consider that these catabolic reactions which take place in many tissues are in some way linked with the hormonal action of the estrogen or other steroid

There are, however certain differences between those vitamins which function as coenzymes and the estrogenic hormones In the first place, the vitamins are absolutely indispensable for life whereas an animal can survive in the absence of estrogens, and the estrogen sensitive tissue is maintained in viable although basal condition in the absence of hormonal stimulation A hypothesis which assigns to the estrogens an obligatory role in a chain of metabolic reactions is difficult to reconcile with the structural variations compatible with estrogenic activity unless conversion to a single compound or multiple and separate sites of action are postulated

An experimental approach to an understanding of the mechanism of action of the estrogens must attempt to link the morphologic alterations which follow the administration of these substances to effects of estrogenic substances at the molecular level

IV Criteria for Experimental Systems

Before discussing the actual experimental approaches to a study of the mechanism of action of estrogenic hormones it seems reasonable to consider criteria which may be applied to an experimental system to determine whether it will give relevant information The goal in mechanistic studies is the development of *in vitro* systems in which reproducible and measurable changes can be elicited by biologically active substances Although it is of course preferable that these systems be derived from tissues which show peculiar sensitivity to estrogens this is not an absolute criterion since most tissues of the body are affected in one way or another by estrogenic substances However, one requirement is that substances which are highly active in the whole animal display activity at low concentrations in the *in vitro* system The matter of concentration is important, since to obtain a convincing effect a concentration must be used which is compatible with that presumed to be present under conditions of estrogen action *in vivo*

In comparing the effective dose of estrogen *in vivo* with the concentra

tions employed *in vitro*, it must be recalled that the amount administered is probably greatly in excess of that required at the sites of action This is a consequence of the rapid rate of inactivation and disposal of administered estrogens

A further consequence of the effectiveness of the inactivation and disposal mechanisms is that an exact parallel between *in vivo* and *in vitro* activity is not to be expected However, substances which are active *in vivo* should display *in vitro* activity It is theoretically possible that substances which are active *in vitro* will be ineffective *in vivo* because they are inactivated before they reach the target site in sufficient concentration If the test system measures estrogenic activity, then compounds which are inactive in the *in vitro* test should also be inactive *in vivo* Futhermore, substances which inhibit the action of estrogens in the whole animal should also be expected to exert their inhibitory effects in the test system, provided they are not first inactivated

These criteria for a study of mechanisms of action of estrogens may not be universally acceptable, but nevertheless they may serve to make clear the basis for the examination of current work in this field The remainder of this discussion will be concerned with the consideration of experimental attempts to discover systems in which the mechanism of action of estrogens may be studied

V *In Vivo* Systems

In vivo systems have been employed first and foremost for the gross and microscopic description of the changes induced in target tissues by the administration of estrogenic hormones These changes are utilized for the development of assay procedures upon which rest the definition and quantitative estimates of estrogenic activity

VI Tissue Culture Studies

Up to this point it has been tacitly assumed that estrogens act directly upon target tissues, and not through other and indirect mechanisms This is almost implicit in the definition of a hormone Evidence in support of this view was provided by the work of Biggers *et al* (1956), who demonstrated a direct action of eleven natural and synthetic estrogens in isolated rings of mouse vaginal epithelium maintained in tissue culture The close correspondence of the stratification and keratinization produced, both *in vivo* and in the tissue culture preparation, provide evidence that (1) alteration of the estrogen molecule in the liver or elsewhere is not a necessary prerequisite for action of the estrogen at the target site (Szego and Roberts 1953, Roberts and Szego, 1953, Fishman, 1951), (2) effects of estrogen are not mediated through the nervous system, and (3) effects of estrogens are independent of blood flow to the target tissue (Hechter *et al*, 1940)

VII *In Vitro* Systems Derived from Estrogen-treated Animals

An important approach to the location of the sites of action of estrogens has been through the comparison of the metabolism of preparations of target tissues removed from control animals with those taken from animals pretreated with physiological doses of estrogens

The Mueller group (Mueller *et al*, 1958) has made an impressive beginning in the examination of the biochemical changes which occur in the uterus of the ovariectomized rat following treatment with small doses of estradiol Within 1 hour of the administration of the steroid, hyperemia occurs (McLeod and Reynolds, 1938) and is followed by a phase of water imbibition which reaches its peak after 4–6 hours (Astwood, 1938, Szego and Roberts, 1953) After another 6 hours true growth begins to occur and a second phase of water imbibition ensues (Szego and Roberts 1953) These changes have been correlated with increases in the protein and ribonucleic acid content of rat uteri In the period from 6 to 24 hours the principal biochemical change observed is an accumulation of ribonucleic acid as measured by uridine thymine ratios in the tissue These conclusions are supported by the observation that the uteri of estrogen treated rats actually incorporate formate C^{14} and glycine 2 C^{14} into the adenine and guanine of nucleic acid When serine 3 C^{14} is incubated with uteri of ovariectomized rats pretreated with estradiol, it is similarly incorporated into the adenine and guanine of nucleic acid—an effect which is blocked by the addition of unlabeled formate to the incubation medium Estradiol has little effect on the labeling of the pyrimidine bases of nucleic acids The failure of estrogen to stimulate the incorporation of $C^{14}O$ into nucleic acid pyrimidines is in contrast to its effect on the incorporation of C^{14} into the acid soluble uridine 5′ phosphate

Attention was also given to the effect of pretreatment with estrogen on protein synthesis in the uterus The incorporation of glycine increased in a linear fashion over the first 12 hour period following pretreatment with estradiol, and reached a peak at about 20 hours Following this, there was a decline, but an increased rate of incorporation could be re established by a second injection of estradiol The stimulation of amino acid incorporation occurred with tryptophan, lysine, serine, as well as with glycine Incorporation of formate into the serine of protein behaved in much the same manner It was further shown, using exchange of P^{3} pyrophosphate with adenosine triphosphate that estrogen pretreatment stimulated amino acid activation This stimulation occurred with leucine, tryptophan, valine, tyrosine, methionine, glycine and isoleucine, but not with thirteen other amino acids which were tested Thus, a certain specificity in effect is manifest

The profound effects of estrogen, both on the cleavage of serine to a one carbon fragment and on the synthesis of serine from formate and gly

cine, suggested attempts to study the effect of estrogen on the level of serine aldolase Pretreatment of the animal with estrogen produced a progressive increase in serine aldolase levels in uterine homogenates fortified with tetrahydrofolic acid These results suggest that an increase either in the amount or in the activity of an enzyme is a consequence of estrogen treatment

Unsuccessful attempts were made to study this effect *in vitro* However, in the presence of 4 hydroxyestradiol there was an irregular effect on serine aldolase activity Uterine segments from ovariectomized rats were then incubated in Eagle's HeLa ultramedium (Eagle, 1955) containing 10% bovine serum and in the presence and absence of estrogen After incubation, homogenates of the segments were examined for their capacity to incorporate glycine 2 C^{14} into protein and for their serine aldolase levels Estrogens did not appear to have any significant effect upon either of these two reactions However, it was found that incubation in this medium produced a remarkable increase in the activity of both test systems when compared with unincubated controls Oxygen was required for this activation which could be abolished either by incubation at 0°C or by the addition of cyanide It was noted that when uterine segments from ovariectomized rats pretreated with estradiol were incubated in Eagle's medium, no further activation was observed It seemed that the effects of pretreatment with estrogen and of incubation in Eagle's medium were similar and approximately additive These observations of a similarity in effect of estrogen pretreatment and incubation in a highly enriched medium may point to a general phenomenon characteristic of estrogen action, namely, the *facilitation of the delivery of the nutritive requirements at a specific site* as a preparation for rapid growth

Other biochemical studies have been concerned with the measurement of enzyme levels in the uteri of castrate rats treated with estrogen over longer periods of time Bever (1959) has reviewed data on the effect of estrogens and other steroids upon the levels of lactic dehydrogenase and reduced diphosphopyridine nucleotide (DPNH) oxidase in rat uteri These experiments were based upon earlier observations that during the estrous cycle the levels of these two enzymes are maximal during early estrus and proestrus and lowest in diestrus and after castration Estrone, estradiol, estriol, progesterone, deoxycorticosterone acetate, testosterone, and cortisone acetate, all tend to elevate the levels of lactic dehydrogenase, the greatest effect being elicited by progesterone and the least clear cut by cortisone acetate In contrast, progesterone in small doses appears to depress the DPNH oxidase activity Progesterone and estradiol are antagonists in both test systems, whereas the other compounds exert lesser inhibitory effects Whether the changes are the expression of activating effects of the hor

mones or the result of altered absolute amounts of enzyme cannot be determined from these experiments

A further example of the effect of estrogens on enzyme activity was reported by Beyler *et al* (1956) who showed that pretreatment of male rats with either estradiol 17β or diethylstilbestrol dipalmitate for a period of 15 days produces a two to fourfold increase in the 3β ol dehydrogenase activity of fortified testis homogenates as compared with controls Even with a dose of estradiol 17β as low as 2 μg per kilogram per day there was a doubling of the enzymatic activity Although the weight of the testes of these animals was not influenced, there was a significant decrease in the weights of both seminal vesicles and ventral prostates as a consequence of estrogen therapy Diethylstilbestrol dipalmitate evoked a similar but less pronounced effect 114 μg per kilogram per day of this substance (equivalent to 40 μg of diethylstilbestrol) producing a doubling in enzyme activity That this effect is not mediated through the pituitary is suggested by the observations of Samuels and Helmreich (1956), who demonstrated a lowering of 3β ol dehydrogenase activity in testicular tissue after hypophysectomy In the present experiments estrogen therapy would be expected to reduce the output of pituitary gonadotropin and thus tend to lower the enzyme level in the testis That this level was increased suggests a more direct effect of estrogen on testicular enzyme activity This paradoxical effect of estrogen, which on the one hand suppresses gonadotropin release, which would depress enzyme activity while on the other hand it increases activity apparently by a more direct effect, is typical of the difficulties which are encountered and must be surmounted in any attempt to explain the mechanism of action of these substances

Interesting effects of estrogen pretreatment upon carbohydrate metabolism in rat liver and the interrelations of estrogen action with adrenocortical secretion and insulin action are discussed by McKerns *et al* (1958) These observations should provide additional points of attack for the development of *in vitro* systems

VIII *In Vitro* Systems

The first clear cut effect of an estrogenic hormone added *in vitro* to an enzyme system was reported by Hagerman and Villee (1953) and Villee and Hagerman (1953) who showed that the addition of estradiol 17β to slices of human endometrium or placenta incubated *in vitro* produced an increased rate of oxidative metabolism In placental homogenates the locus of action of estradiol was considered to be a diphosphopyridine nucleotide linked isocitric dehydrogenase present in the soluble fraction (Villee and Gordon 1955) Triphosphopyridine nucleotide did not replace diphosphopyridine nucleotide (Villee, 1955), and a divalent metal cation is required for full

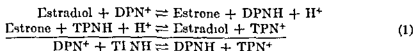
activity (Gordon and Villee, 1955) The data are consistent with the view that estradiol combines with an inactive form of the enzyme, thereby rendering it enzymatically active A kinetic analysis (Gordon and Villee, 1956) provides the basis for the enzyme activation concept When the steroid is the limiting reagent in the assay system the formation of reduced diphosphopyridine nucleotide can be used as an assay for estrone and estradiol 17β

When the effect of various steroids upon the reduction of DPN by placental extracts was studied, it was found that estrone and estradiol had approximately the same activity Estriol at fifty times the concentration had no effect, while 17α ethynylestradiol had slightly more than one third the effect of estradiol Neutral steroids, with the exception of testosterone, were essentially inactive In the case of testosterone, however, there was a significant reduction of DPN which may be attributable to a soluble testosterone dehydrogenase which was found in human placenta by Meyer (1955) Diethylstilbestrol was in these experiments inactive, as was estradiol-17α It is noteworthy that a mixture of 0 3 μg of estradiol and 12 μg of estriol produced a significantly smaller reduction of DPN than did the same amount of estradiol alone A mixture of estradiol with progesterone or cortisol had no effect, whereas testosterone appeared to enhance DPN reduction The position of diethylstilbestrol in this series is a little puzzling since it is an exceedingly effective estrogen in the whole animal

Hagerman and Villee (1957) measured the dissociation constant for estradiol 17β and estrone and the estrogen sensitive enzyme The two natural estrogens are bound to the enzyme about 200 times more strongly than is diethylstilbestrol, the latter compound has only half as great an ability to activate the enzyme When estradiol and diethylstilbestrol are both present, the latter acts as a competitive inhibitor A parallelism between the effects of estrogens in activating the enzyme and their action in the whole animal was supported by tests (Villee and Hagerman, 1957) on a series of aromatic anti estrogens of which some were higher homologs of diethylstilbestrol (Bárany *et al*, 1955) Diethylstilbestrol, 1,3 di(p hydroxyphenyl)propane, and estradiol 17α were found to be competitive inhibitors of the activation of placental isocitric dehydrogenase by estradiol In addition it was found that estriol alone has a slight activating effect but combined with estradiol it decreases the activating effect of the hormone These findings were interpreted as indicating that estrogens and substances related to them have two distinct properties the first being to combine with the enzyme, and the second, to activate it The experiments on homologs of stilbestrol indicated further that the size and the shape of the molecule are important in determining binding to the enzyme surface

The observations of Villee and his co workers have recently been confirmed and extended by Talalay and Williams Ashman (1958), who found that in more highly purified preparations of human placenta the estrogen effects disappeared but could be restored by the addition of catalytic amounts of triphosphopyridine nucleotide (TPN) The effect of TPN on the reduction of DPN was manifest only in the presence of both substrate (isocitrate) and estradiol These observations were interpreted as indicating an effect of the hormone not upon isocitrate dehydrogenase but upon a transhydrogenase system Further support was provided by the observation that isocitrate dehydrogenase could be replaced by glucose 6 phosphate dehydrogenase Thus, the locus of action of estrogen was shifted from isocitrate dehydrogenase to a transhydrogenase system The similarities in properties and kinetics of the transhydrogenating system and the estradiol dehydrogenase of human placenta described by Langer and Engel (1958) led Talalay to conclude that these enzymes were identical

It suggested that the reversible oxidation and reduction of the steroids themselves constituted a steroid activated transhydrogenase system according to equation (1) The net effect is the transfer of hydrogen from TPNH



$$\begin{array}{l} \text{Estradiol} + DPN^+ \rightleftharpoons \text{Estrone} + DPNH + H^+ \\ \underline{\text{Estrone} + TPNH + H^+ \rightleftharpoons \text{Estradiol} + TPN^+} \\ DPN^+ + TPNH \rightleftharpoons DPNH + TPN^+ \end{array} \qquad (1)$$

to DPN This concept would place the steroid hormone in the position of a coenzyme which controlled the flow of electrons into the two sets of metabolic reactions in which di and triphosphopyridine nucleotides play their specific roles Implicit in this concept is the identity of the DPN linked estradiol dehydrogenase the TPN linked estradiol dehydrogenase, and the transhydrogenase These experiments linking TPN with a possible biochemical function for estradiol have certain points of contact for the work of Mueller cited above since Flaks *et al* (1957) and Huennekens *et al* (1957) have demonstrated that TPN is required for the incorporation of one carbon fragments into serine and into purines

The experimental observations of Talalay and Williams Ashman were soon confirmed by Villee and Hagerman (1958), who presented additional evidence for the view that the site of action of estradiol was actually the transhydrogenase system by demonstrating the coupling of transhydrogenation with a TPN linked *yeast* isocitrate dehydrogenase (Talalay and Williams Ashman used a rat heart enzyme) and with glucose 6 phosphate dehydrogenase measuring in the latter case relative amounts of $C^{14}O_2$ produced from glucose 1 C^{14} and glucose 6 C^{14} as well as reduced pyridine nucleotide formation

Attempts to couple the transhydrogenation with lactic dehydrogenase

derived from either yeast or liver, or with alcohol dehydrogenase, we successful, thus indicating some degree of specificity in the coupling transhydrogenation reaction. It was also found that the addition of es did not affect the rate of the reaction of lactic dehydrogenase or dehydrogenase with their substrates in either direction. Finally, by v the relative amounts of di- and triphosphopyridine nucleotide add purified placental preparations in the presence of excess isocitrate, shown first, that in the presence of catalytic amounts of TPN and sub amounts of DPN, there was a pronounced estrogen effect. However, amount of TPN was increased, the estrogen effect diminished, and presence of substrate amounts of TPN no estrogen effect was ma These experiments, too, are completely consistent with the view th locus of action of estrogen is the transhydrogenase. However, the pr is raised by the authors that such compounds as bisdehydrodoisynoli and diethylstilbestrol are indisputably estrogens and yet do not fit in mechanism proposed by Talalay and Williams-Ashman.

The principal support for the Talalay concept that the estrogens fu as hydrogen transfer agents rests on the supposition that the estradi hydrogenase described by Langer and Engel (1958) is identical wi transhydrogenase. In a recent paper (Talalay *et al.*, 1958) evidence i sented that during the course of a 200 fold purification of the DPN l dehydrogenating activity in human placenta, there was a parallel pu tion of the estradiol specific transhydrogenase when it was assayed procedure which corrects for estradiol insensitive transhydrogenation fication of the enzymatic activities from term placenta was accomp by removal of the microsomes with calcium chloride, followed by a nium sulfate fractionation, heat treatment, and a second ammonium s fractionation. The ratio of dehydrogenase activity to estradiol sp transhydrogenase activity increased from 2.3 in the supernatant fra from the calcium chloride precipitation to 9.7 in the fraction precipi by 20–25% ammonium sulfate in the final step. At the same time th hydrogenase activity increased from 66 units per milliliter to 850 unit milliliter, whereas the estradiol specific transhydrogenase activity cha from 29 to 88 units per milliliter. The highest content of both dehydrog and transhydrogenation activities was found in the 10–15% ammo sulfate fraction and had a ratio of dehydrogenase transhydrogenase o Although the authors assert that there was no tendency for separati these two activities over a purification range of 100 fold, the ratio of ac ties does change, and there is a fourfold increase from the supernatant tion from calcium chloride precipitation to the 20–25% ammonium su fraction. The problem of the separate natures of the transhydrogenase dehydrogenase now appears to have been resolved by more recent wo

Hagerman and Villee (1959) and Hagerman *et al* (1959) These authors have shown that the dehydrogenase and the transhydrogenase are inhibited by sulfhydryl reagents at different rates, are thermally inactivated at different rates, and that the ratio of dehydrogenase to transhydrogenase activity does not remain constant during purification Finally, by the use of electrophoretic techniques three enzymatic activities have been separated, a DPN linked estradiol dehydrogenase, a TPN linked estradiol dehydrogenase and an estradiol sensitive transhydrogenase No transhydrogenation occurs when the two dehydrogenases are mixed in the presence of estradiol

The present position appears to be that although the locus of action of estrogen is upon a transhydrogenase, as first suggested by Talalay, the estradiol presumably does not exert its effect by functioning as a coenzyme in the simple manner postulated by him The mechanism by which it does act is unknown and is merely redescribed as "activation" It will be recalled that this term was first introduced by Gordon and Villee (1956) The "activation' mechanism permits greater structural latitude for estrogenic activity than does the postulated coenzymatic role The latter requires the presence or the facile formation of a reversibly oxidizable alcohol group Thus, the potentiating effects upon the transhydrogenase system of bisdehydrodoisynolic acid (Villee and Hagerman, 1958) and 17α ethynylestradiol (Gordon and Villee 1956) can be accepted by the 'activation theory but are difficult to reconcile with the coenzyme mechanism

Further information concerning the structural specificity of the transhydrogenase system has been reported by Hollander *et al* (1958a) who studied the effect of variations in structure of estradiol derivatives upon α ketoglutarate production in the transhydrogenase system coupled with isocitric dehydrogenase The results in general are well correlated with the substrate specificity studies on estradiol dehydrogenase (Langer *et al* 1959) carried out on a more extensive series of compounds 17α Ethynylestradiol again was noteworthy since at a concentration of 10 μg per milliliter it was approximately one third as effective as estradiol in stimulating the reaction The parallelism between the structural requirements for participation in the transhydrogenase reaction and ability to serve as a substrate for the DPN linked dehydrogenase seem at first sight to support the concept of a coenzymatic role for the steroid although again ethynylestradiol strikes a discordant note However it is as reasonable to interpret these findings as signifying that the receptor binding sites which operate when estrogens exert their potentiating effects upon the transhydrogenase protein are as closely related to the total structure of the estrogen molecule as are the binding sites on an enzyme protein for which the estrogen is a substrate

In assessing these results, several other factors must be kept in mind First, we have as yet no information concerning the quantitative importance

of the estrogen activated transhydrogenase in the total economy of the cell Secondly, the phenomenon has been studied most thoroughly in placenta a tissue which synthesizes estrogens in relatively abundant quantities and which exists throughout its life in an environment rich in estrogens However, further emphasis is given to this phenomenon by the fact that estrogen sensitive transhydrogenase systems occur in other estrogen targets, namely, endometrium (Hagerman and Villee, 1953) and breast (Hollander *et al* , 1958b, 1959) These observations are consistent with the view that the estrogen sensitive transhydrogenase is related to the hormonal actions of these substances, but are by no means proof

It should be expected that there are probably other mechanisms and sites of action for the estrogens, some of which may require intact cells

IX Summary

One may perhaps summarize the present position in regard to mechanisms of action of estrogens as follows the description of the phenomena at the gross and microscopic level is well established If the demonstration of estrogen effects in tissue culture serves to establish the fact that these substances act locally upon the tissue affected and are not mediated through any systemic mechanisms, there is then a middle ground which consists of the descriptions of changes in enzyme content and activities in the susceptible tissues of pretreated animals This type of information serves as the means of discovering *in vitro* systems in which estrogen effects may be observed

Finally, at the level of enzymatic reactions, the effect of estrogens upon the transhydrogenase system present in several estrogen sensitive tissues has provided the springboard for speculations concerning the intimate mechanism of action of these substances It would be premature to say that we had any real understanding of these mechanisms, but the widespread interest evoked in this area will undoubtedly lead to a rapid accumulation of information and a deeper insight into this problem

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I Introduction

In recent times much knowledge has been gathered on factors that play, or may play, a role in the etiology of atherosclerosis Besides other studies, many investigations of the blood plasma constituents have been made to establish their possible relationship with atherosclerosis, and also to find the relation of these blood constituents, and of atherosclerosis, to such factors as diet, habits of life, race, and climate

In a similar way some of these techniques have been used for studies of pregnant women, and, less frequently, in cases of complicated pregnancy Of the latter, only data on toxemia of pregnancy (pre eclampsia, eclampsia) are considered here

In the literature on both subjects—especially recent papers—there can be found a surprising number of proved or suggested causes and biochemical deviations from the normal which are the same, or appear to be very similar, both in atherosclerosis and toxemia of pregnancy Most of the work done on atherosclerosis has been in relation to coronary sclerosis

Evidence for correlations between the two diseases should be sought for by comparing the data on the diseased subjects with the corresponding normal ones, i e subjects with coronary sclerosis compared with healthy persons of the same sex and age, and patients with a toxemia of pregnancy with women with uncomplicated pregnancy of comparable duration

The available data can be divided into the following main groups

1 (a) Dietary factors suggested or proved to play a role in causing or preventing the disease in human beings, either by a deficiency or by excess of one or more substances (b) Dietary measures causing the same or very similar disease in experimental animals, together with measures to prevent the development of the disease in these animals

2 (a) Deviations from the "normal" of the blood plasma levels of a number of substances, whether normally present or not This includes also reactions occurring in the blood or blood vessel walls the underlying abnormalities of which are not yet known (b) Deviations from the "normal" amounts of substances excreted by the kidney, including data on substances present only in the diseased

3 The influences of nondietary therapeutic measures

4 Habits of life which can play a role in inducing or preventing both diseases

5 Possible similarities in the clinical symptoms and the (anatomical) pathological findings in both diseases, and their course with time

Before discussing the above mentioned points in detail, it should be noted that only those factors are recorded on which some information could be found bearing on *both* diseases or data of more general importance There are of course 'factors' the roles of which have been described in only one of the diseases These have been omitted

II DIETARY FACTORS THAT PLAY A ROLE IN HUMAN BEINGS

Many papers have been published in recent years on the influence of diet upon atherosclerosis and especially coronary sclerosis (Welch *et al*, 1958) Data on the relation between diet and toxemia of pregnancy are not as numerous and many are of somewhat older date

1 General Evidence

The influence of 'civilization' can be mentioned as general evidence for a positive correlation of dietary factors with these two diseases Civilization has proved to be unfavorable in both diseases In dietary terms "civilization" can largely be seen as the possibility of obtaining preserved, canned, shortened, and refined foods, possibly combined with less need for physical activity in obtaining food, and consequently a lower need for calories

Extensive studies on the world distribution of both diseases have been carried out—by Keys (1953), Keys and Anderson (1957), Keys *et al* (1957a, b) Brock *et al* (1956) Brožek *et al* (1957), Bronte Stewart *et al* (1955), Enos *et al* (1955) Mann, (1957) and Henschen (1953) in the field of coronary heart disease, by Whitacre (1954) Bell (1956) Bell and Wills (1955), Hipsley (1953), De Snoo (1949), Scrimshaw *et al* (1947), and Park and Dawson (1952) as to toxemia—in which the influences of race sex and climate are taken into account together with variations and changes in dietary habits, and also the migration into other countries of large numbers of people of the same origin

Unfortunately the frequency of both diseases has never been measured intentionally at the same time in the same population group There are only the data of Duncan (1947), concerning a Subarctic region and the data obtained during the world wars Fortunately, data on both diseases measured in the Jamaican population were recently given by Tulloch (1958) Robertson (1958), and Robertson and Dixon (1958) Moreover it is extremely difficult to obtain comparable data According to the literature mentioned a high frequency of toxemia has been recorded in Western Europe, Canada (Toronto), the United States including Negroes, Sydney, Austrialia, New Zealand, Indians in southern India and Colombo, Indians in the Fiji Islands, etc A low incidence has been reported among native Fijians, in the native New Guinea and Solomon Island population, in

Jamaica, the Punjab, Java, Celebes, in the primitive Negro population in Africa, in the Subarctic, and among Eskimos In general, the incidence seems to be rather similar to the distribution of coronary mortality, but no proof can be given as yet

Another general influence is that of the world wars A decrease in the frequency of both diseases has been seen in all European countries where food was rationed, with a subsequent increase after the normal food supply was restored[1] (for atherosclerosis see Malmros, 1950, Strøm and Jensen, 1951, Keys, 1953, Groen and van der Heide, 1956a, b, Breirem 1957, and for toxemia of pregnancy, Holmer, 1947, Mastboom, 1948, Van Bouwdijk Bastiaanse, 1954, Hilber and Mentz, 1954, Burger, 1950, and Taymergen, 1955)

As to toxemia of pregnancy it might be suggested that the reduced frequency was partly due to extra food rations, for instance, milk and vitamin supplements given to pregnant women But on the other hand, the sudden greatly increased availability of food after the liberation produced at once a much higher frequency (Holmer, 1947, Jung, 1947) This may be compared with the evidence of Kyhos *et al* (1949) obtained in Naples among the poor, in whom the toxemia incidence rose on "improvement"—qualitatively and quantitatively—of the diet A similar argument is that the toxemia cases seen during the war occurred mainly in those who had better possibilities of obtaining food, for instance the wives of black marketeers

In any case, the food consumed during the war had much in common with the food of primitive people it was less refined, contained less animal fat and proteins, more fruit and vegetables, whether fresh or not, and was in general, a low fat, and often a low calorie, diet distinctly richer in vitamin and mineral content than at other times

Of course, the food supply can be actually insufficient during war and famine, and a corresponding increase in the mortality rates can be expected as a result It is quite conceivable that this might affect pregnant women more than other subjects

As to pregnant women, a number of surveys have been made in which dietary supplements were given based on the knowledge obtained during war time, for instance, the Toronto experiment (Ebbs *et al*, 1942a, b), the London survey (Peoples League of Health Report, 1942 1946), the Vanderbilt study (Darby *et al*, 1953a b, 1955), and many others (Mellanby, 1929, Theobald, 1937, Sinclair, 1944, Baird, 1944 reviewed by Magee,

[1] The same trends were present for dental caries diabetes and the frequency of thrombosis and embolism (Toverud 1956 1957, Malmros 1950 Boerema 1947)

[2] By primitive people is meant those living in their natural state from the natural food sources not those civilized just enough to earn nearly no money and buying only the cheapest staple Western foods These last groups often live in very poor conditions and suffer from many diseases

1946) In general, favorable results were seen, but how little actually is known on the subject was shown by the Neapolitan work of Kyhos *et al* (1949), already mentioned, in which the original diet was qualified as very poor, yet the toxemia incidence proved to be low Toxemia incidence actually rose on supplementing the diet with ingredients considered to improve the diet in quality and quantity Similar results to those of Kyhos had already been reported by other authors Moreover, although primitive people often consume diets which are considered to be very poor, they have low mortality rates of atherosclerosis as well as a low toxemia incidence in general (see for instance, Bronte Stewart *et al* , 1955, Holemans and André, 1955 Duncan, 1947, Tulloch 1958, and Robertson, 1958)

A third general argument for a positive relation between the frequency of the two diseases is the possibility of inducing the diseases in experimental animals by a number of closely related dietary measures for each disease Details are given below in Section III The above mentioned reports concern rather large and multiple changes in the composition of the diet, surveys with only one or two factors involved will be discussed below

The dietary factors studied in detail have been amount of calories ("over weight" or not), refining of foods fruits and vegetables, fats, proteins, carbohydrates cholesterol vitamins A D (cod liver oil) E, and B (especially niacin and pyridoxine) choline cystine methionine, and minerals (copper magnesium sodium potassium) In the following discussions the disease for which the best documentation was found is usually described first

2 Calories

Investigations of the caloric level of diets point immediately to a link between "overweight" and disease According to Keys and Anderson (1954) Keys (1955) Keys *et al* (1955) and Anderson *et al* (1957a, b), there is no direct relation between the body weight and the cholesterol level of the blood whereas the average cholesterol levels of certain population groups have a direct relation of the coronary mortality rates in these groups

In the active state of developing obesity by increasing the calorie intake the serum cholesterol tends to rise but this increase is not maintained when weight gain ceases Weight loss gives a decrease of the cholesterol level, the effect is greater when the reducing diet is low in fat than when it is high in fat (Anderson *et al* , 1956) On the other hand people rapidly losing weight may have elevated cholesterol levels

Wartime rationing causing a considerable caloric restriction resulted in most cases in lower cholesterol levels, while the frequency of both athero sclerosis and toxemia of pregnancy was reduced Obviously the qualitative changes in the diet cannot be neglected, and the reduced intake of animal

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fats may have had an especially important role, apart from its caloric value Moreover, Schmidt (1955) remarks that in all countries where the blood cholesterol values are low, calorie intake is low too

The primitive Eskimo and Lapp consume a fairly high fat diet [the diet is not necessarily rich in fat (see also Duncan, 1947)], but famine occurs sometimes as a result of the migration of animals and fish from the areas where these primitive people live (Scott, 1956) Greater physical activity and concomitant greater energy expenditure will be important in these cases, as (partly) during war time

A suddenly improved supply of foods such as occurred at the end of the war in Western Europe resulted in an immediate sharp increase of the toxemia incidence The incidence of coronary mortality rose likewise, but not so sharply This difference in rate of increase is not as contradictory as it might seem, since it might well be related to the acute course of toxemia of pregnancy in comparison with the always more chronic progress of coronary sclerosis

The effect of a sudden great improvement—qualitatively and qantitatively—of a very poor diet has also been seen by Kyhos *et al* (1949) in pregnant Neapolitan women The toxemia incidence, which had been low before the diet was supplemented, rose significantly The weight increase induced by these dietary measures will have caused a greater tendency to an increase of the cholesterol level, and it is known that in toxemia of pregnancy cholesterol levels are elevated above the values in normal pregnancy (see Section IV, 1)

Williams reported recently (1957) that toxemia patients had a higher average initial weight and a greater weight increase (partly due to edema) than have normal pregnant women Dieckmann (1952) calculated that a normal pregnant woman should not have a weight increase of more than 6 7 kg A weight increase of more than 8 kg increases the danger of pre eclampsia Others, however, tolerate a weight increase of about 10 kg, for instance 9 5–10 5 kg (Williams, 1957) Thomson and Billewicz (1957), on the other hand, consider 12 5 kg a normal amount In comparison with the weight increase records of primitive people (in whom toxemia is rare) this seems too high (Holemans and André, 1955, Lambillion, 1950, Geber and Dean, 1957), however, race influences play a role also Hamlin *et al* (1953) reported a reduction of the eclampsia incidence from approximately 1 in 500 to 1 in 7000 by avoiding undue weight increase by strict attention to the diet The whole invesigation covered 18 000 cases in a Sydney hospital Standard antenatal care during 1935–1947 resulted in eclampsia in 106 of 37,000 cases After starting weight control from earliest pregnancy in 1948, the frequency of pre eclamptic albuminuria was lowered from 10 to 1 8% and booked eclampsia went down to the level already mentioned

of 1 in 7000 at the end of 1951 [3] The higher risk of toxemia of pregnancy in obese women—defined by initial weight as well as by weight gain—has been reported by other authors also (see for instance Theobald, 1955, Thomson and Billewicz 1957, Darby *et al*, 1953a, b, 1955, and McGanity *et al* 1954, 1955, in the Vanderbilt study)

An increased weight postpartum has been reported repeatedly as occurring frequently in countries with a known high incidence of toxemia, whereas this was not seen, for instance, in the studies of Holemans and André (1955) in the Belgian Congo

3 *Refinement of Foods*

The refinement of foods has many aspects and is not limited only to one or two important dietary factors Most of the relative deficiencies mentioned in this paper are certainly partly due to refining and preserving of food stuffs and the preference for these foods, considering taste as well as economic factors

There is, however, one special constituent of nonrefined foodstuffs that has been extensively studied by Hipsley (1953) and by Bell and Wills (1955) These authors compared the fiber contents of the diets from a large number of studies in different parts of the world They noticed that the women in population groups consuming diets with high fiber content suffered less from toxemia whereas shifts in the dietary composition in one or another direction changed the toxemia frequencies in concordance with this view

Except for the war reports, there are no data obtained simultaneously on the incidence of atherosclerosis As has been already remarked, both diseases were less frequent in Europe during the last world war

A high fiber content of a diet always means a high intake of fruits and vegetables and such a diet will give a higher intake of vitamins and minerals trace minerals as well as others, and possibly other unknown factors Further the intake of vegetable fat is relatively high and the intake of animal fat is relatively low while in general, the total fat intake is also

[3] A toxemia incidence of 13% was seen in an Amsterdam investigation carried out in 1950-1951 (Van Bouwdijk Bastiaanse *et al* 1959) covering nearly 500 women Toxemia was diagnosed here when the blood pressure was over 140/90 on one or more occasions during the prenatal examinations (before the eighth month of pregnancy) In another investigation (Nederveen Fenenga *et al* 1959) a few years later (1955) also in Amsterdam about 25% of all women studied had salt restriction prescribed from the sixth month of pregnancy or earlier However in one of the Amsterdam clinics it was noticed accidentally (spring 1957) that a rather small percentage of women escaped the prescription of saltless regime during the last weeks of pregnancy also However these data are not completely comparable and especially in the last one the physicians concerned might have been overcautious

low The bulky content of such a diet will also discourage the intake of excess calories and thus unwanted gain of weight In addition, loss of calories by incomplete absorption in the intestine can play a role (incomplete digestion of fiber containing food particles and increased bowel activity)

4 Fats

The exact role of dietary fats and the relation of the essential fatty acids in this respect are still under discussion From the studies of Keys *et al* (Keys, 1953, 1955, Keys and Anderson, 1954, 1957, Keys *et al*, 1955, 1956a, b, 1957a, b, c, d, 1958, Anderson *et al*, 1956, 1957a, b) it is known that a definite correlation exists between the prevalence of coronary heart disease and the fat content of the diet Population groups consuming diets low in fat have a low average cholesterol level of the plasma and a low frequency of coronary heart disease On the contrary, high fat diets promote high average cholesterol levels and a high frequency of coronary disease In general the low fat diets are low in animal fat and thus most of the fat is of vegetable origin (see also Anderson *et al*, 1957a, Brožek *et al*, 1957, Fidanza *et al*, 1957, Ferber and Buzina, 1957) Moreover, it is known that the ingestion of vegetable and marine oils with a high content of unsaturated fatty acids produces a fall in the level of plasma cholesterol, especially in subjects with high initial cholesterol levels Animal fats and oils with a low iodine number have a reverse effect and tend to increase the cholesterol level in man (Beveridge *et al*, 1956, Bronte Stewart *et al*, 1956, Brock *et al*, 1956, Ahrens *et al*, 1957, Keys *et al*, 1957a, b, c, Kinsell, 1954, 1956, 1958, Kinsell *et al*, 1953, 1958, Malmros and Wigand, 1956, 1957) Aftergood *et al* (1956) showed the same thing in rats However, causes and effects have not yet been resolved

Data on the clinical effects of fats, apart from the influence on the blood lipid system, are scarce The possibility of prevention of coronary heart disease by changing the intake of the type and amount of fats cannot be given in percentages, and data on clinical improvement are extremely difficult to evaluate since subjective factors play such an enormous role This last argument is much less important in cases of toxemia of pregnancy Very recently Rutstein *et al* (1958) demonstrated the essential role of the cholesterol present in the culture medium, in intracellular lipid deposition by tissue cultures of human aortic cells The lipid deposition was inhibited by adding linolenic acid to the cholesterol containing culture medium and was increased by adding stearic acid to this medium

According to Gordon *et al* (1957) the unsaturated fatty acids lower the plasma cholesterol in man by an increased conversion to bile acids and fecal excretion of the latter Lewis (1958) confirmed this in studies in three pa

tients with a complete bile fistula Feeding sunflower seed oil (by mouth) and cottonseed oil (intravenously), which are both highly unsaturated, produced a remarkable increase in the rate of cholic acid secretion, this increase preceded the fall in the serum cholesterol level A saturated fat (hydrogenated coconut oil) did not affect the bile acid output, but raised the serum cholesterol Hellman *et al* (1957), however, working with C^{14}-labeled cholesterol, observed a decreased output of cholesterol and its end products on feeding butterfat and an increased excretion on corn oil feeding to a hyperlipemic patient Gottenbos and Thomasson (1958) demonstrated that the fecal excretion of cholesterol is higher in rats on corn oil diets than in those on coconut oil diet They suggested that the cholesterol is excreted by the bowel wall

Although papers concerning the influence of fats and oils in atherosclerosis or coronary heart disease as well as on "healthy" subjects, are far too numerous to mention in full, data concerning toxemia of pregnancy and the possible role of fats are very scarce indeed Garret (1951) considers an imbalance of dietary protein fats, and carbohydrates to be a primary cause of eclampsia According to his experience, dietary fats and particularly lard and other pork products should be restricted for the prevention of toxemia Silbernagel and Patterson (1951) reported a favorable effect from wheat germ oil—one of the oils which is very effective in decreasing blood cholesterol levels—on the incidence of toxemia of pregnancy In the control series of 1973 women 10% developed toxemia In the 825 women who were given three times a day a 3 minim capsule of an eight times concentrated preparation only 2 1% showed evidence of toxemia, 90% of the subjects started therapy between the third and sixteenth week of pregnancy

Stamler (1956) described a diet which causes a toxemia of pregnancy in rats The effect disappeared when corn oil was given instead of cod liver oil Crude linoleic acid was approximately as effective The eclamptic disease could be prevented by prior treatment of the test animals with vitamin E (α tocopherol) or lettuce or by adding these substances to the toxic diet

Especially this last experiment again points to the important metabolic relations between vitamins E and D and the unsaturated fatty acids including both the 'essential' and the "nonessential" ones Some may even be toxic as is probably the case in cod liver oil It is known that a high intake of unsaturated fatty acids increases the need for vitamin E, possibly because there is more need for antioxidant The need for vitamin A has been said to increase as well There are also relations with pyridoxine, which is necessary in the production of arachidonic acid from linoleic acid etc (Leitner, 1957, Hove and Harris, 1951 Hove, 1955, Sinclair 1956,

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According to Gordon *et al* (1957) the unsaturated fatty acids lower t plasma cholesterol in man by an increased conversion to bile acids and fe excretion of the latter Lewis (1958) confirmed this in studies in three p

tients with a complete bile fistula Feeding sunflower seed oil (by mouth) and cottonseed oil (intravenously), which are both highly unsaturated, produced a remarkable increase in the rate of cholic acid secretion, this increase preceded the fall in the serum cholesterol level A saturated fat (hydrogenated coconut oil) did not affect the bile acid output, but raised the serum cholesterol Hellman *et al* (1957), however, working with C^{14} labeled cholesterol observed a decreased output of cholesterol and its end products on feeding butterfat, and an increased excretion on corn oil feeding to a hyperlipemic patient Gottenbos and Thomasson (1958) demonstrated that the fecal excretion of cholesterol is higher in rats on corn oil diets than in those on coconut oil diet They suggested that the cholesterol is excreted by the bowel wall

Although papers concerning the influence of fats and oils in atherosclerosis or coronary heart disease, as well as on "healthy" subjects, are far too numerous to mention in full, data concerning toxemia of pregnancy and the possible role of fats are very scarce indeed Garret (1951) considers an imbalance of dietary protein fats, and carbohydrates to be a primary cause of eclampsia According to his experience, dietary fats and particularly lard and other pork products should be restricted for the prevention of toxemia Silbernagel and Patterson (1951) reported a favorable effect from wheat germ oil—one of the oils which is very effective in decreasing blood cholesterol levels—on the incidence of toxemia of pregnancy In the control series of 1973 women 10% developed toxemia In the 825 women who were given three times a day a 3 minim capsule of an eight times concentrated preparation, only 2 1% showed evidence of toxemia, 90% of the subjects started therapy between the third and sixteenth week of pregnancy

Stamler (1956) described a diet which causes a toxemia of pregnancy in rats The effect disappeared when corn oil was given instead of cod liver oil Crude linoleic acid was approximately as effective The eclamptic disease could be prevented by prior treatment of the test animals with vitamin E (α tocopherol) or lettuce or by adding these substances to the toxic diet

Especially this last experiment again points to the important metabolic relations between vitamins E and D and the unsaturated fatty acids including both the essential' and the 'nonessential" ones Some may even be toxic as is probably the case in cod liver oil It is known that a high intake of unsaturated fatty acids increases the need for vitamin E, possibly because there is more need for antioxidant The need for vitamin A has been said to increase as well There are also relations with pyridoxine, which is necessary in the production of arachidonic acid from linoleic acid, etc (Leitner, 1957, Hove and Harris, 1951, Hove, 1955 Sinclair, 1956,

Irving and Budtz Olsen, 1955, Irving, 1956, Luttrell and Mason, 1949, Filer *et al*, 1946, Day and Dinning, 1956)

Recently Holman (1958) discussed all known roles of the essential fatty acids He points out again that the lipids of the gonads contain an unusually high proportion of the polyunsaturated fatty acids and that pregnant female rats on diets deficient in essential fatty acids show impairment of reproduction The blood phospholipids and the cholesterol esters also contain a high proportion of unsaturated fatty acids

A few general remarks may be made at this point The bacon industry asks for hogs with a hard fat This fat is low in content of essential unsaturated fatty acids From the medical point of view it is certainly interesting and important to know that the composition of the carcass fat of this animal strongly reflects the type of fat, or better of fatty acids, taken in its food The fat of hogs fed a soybean oil diet may contain as much as 39% linoleate (Sinclair, 1957) and thus might well be a good source of essential fatty acids, perhaps without an elevating effect on the cholesterol level of those who consume it Hogs grown on a diet low in fat and high in carbohydrate store a harder and more saturated fat The tissue fat deposited in hogs on such a diet contains about 70% of the C_{18} acids oleic and stearic The content of linoleic and the other poly unsaturated fatty acids in the fat is largely dependent on the oils given in the rations (Ellis and Hankins, 1925, Ellis and Ishbell, 1925, Ellis and Zeller, 1930, Hilditch *et al*, 1939)

Furthermore, partial hydrogenation of fats as applied in the food industry may cause isomerization so as to produce isomers which may have an adverse effect on the cholesterol level and may thus cause higher instead of lower levels However, there is no proof that this is so

5 Proteins

A large total amount of protein has often been described as of great importance in preventing toxemia of pregnancy (see e g, Arnell *et al*, 1945, Burke, 1945, 1950) On the other hand, many primitive people consume little protein, and yet their toxemia incidence is not high Under war conditions the toxemia incidence was distinctly lower during World War II than before and afterward, and the protein intake was certainly lowered There are also a number of nutritional studies in which no influence of the dietary protein content on the frequency of toxemia could be seen For instance, in the Vanderbilt study (McGanity *et al*, 1954, Darby *et al*, 1955) there was no difference between the toxemia incidence in women consuming more than 50 gm of protein a day and those consuming less than 50 gm Dieckmann *et al* (1951) also could not find any influence of the protein intake

A rather similar situation exists for atherosclerosis, at least as far as the cholesterol level of the plasma is concerned Keys *et al* (1957a-d) did not

see any influence on the cholesterol levels of large differences in the dietary protein supply (from 8 to 20% of total calories), whether or not a low or a high fat diet was given to the subjects Olson *et al* (1957) reported a lowering of the serum cholesterol in man on decreasing the protein content of the diet De Groot (1958) observed that an increased amount of protein in the diet of rats decreased the cholesterol levels The activities of the proteins tested were, in decreasing order wheat gluten, casein, gelatin

6 *Carbohydrates*

Very little is known about a possible role of carbohydrates in these diseases, only a few general remarks can be made that may be important Excess carbohydrates are converted into fat, which in turn is deposited in the tissues The fatty acids produced in this way are of the saturated type, or may have one double bond Quantitatively the most important are palmitic stearic, and oleic acid The higher unsaturated fatty acids can not be produced from sugars Laurent (1958) recently reported a very marked elevation of plasma triglyceride when carbohydrate is substituted for fat in the diet, but no effect on the cholesterol level was seen, indicating an acceleration of lipogenesis

In this connection it is important to note the data of Nickols *et al* (1956), who observed that an increased carbohydrate intake without an increase of the caloric intake may cause a rise of the level of the S_f 20–400 lipoproteins in some people These lipoproteins are closely related to the chylomicrons, which contain much fat The cholesterol level showed no marked change, and the effect was not influenced by the fat consumed

An inverse relation between the lipoprotein levels and the blood sugar levels was found by Wolff and Salt (1958) in diabetic children, there was also an inverse relation between total lipid as well as cholesterol levels and the glucose level

As a shift in the lipoprotein values in the same direction as occurs in atherosclerosis has been seen in toxemia of pregnancy, these data may be important for both diseases

It should be kept in mind also that latent diabetes may turn into real diabetes during pregnancy and become latent again after parturition during another period of time Diabetic women have a marked inclination to develop toxemia of pregnancy as well as atherosclerosis (Sindram, 1955) The toxemia may however be a sequel of the already developed vascular changes as well as of new vascular changes and/or other causes of placental hypoxia

7 *Cholesterol*

The cholesterol of the diet has been proved not to be the factor in causing high blood cholesterol levels Keys *et al* (1956a, b) demonstrated in a

number of surveys that there is no relation between the cholesterol content of the diet and the cholesterol level in the plasma Even doses of 10 gm of cholesterol per meal give only a very slight and temporary increase of the blood cholesterol level According to these authors, the Sardinians, for instance, who consume a diet that may have large individual differences in cholesterol content, owing to the consumption of 0 to 30 eggs, invariably have a low blood cholesterol level

Administration of extra cholesterol to pregnant women has been carried out by Moses *et al* (1952) A dose of 2 gm a day during the last four months of pregnancy had no influence on the plasma cholesterol, the cholesterol ester, or the lipid phosphorus content compared with a control group As the cholesterol level in toxemia of pregnancy is still higher than the already elevated level in uncomplicated pregnancy it is important to know that this level, too, is not induced by a high cholesterol intake (see also Section IV, 1 on changes in blood) The blood cholesterol level thus seems to be influenced remarkably little by exogenous supply in view of the daily intake in relation with the total amount present in the plasma However, the presence of certain oils in the diet can play a role, and the possible effects of surface tension depressing substances should be given special consideration (see also the review by Felch *et al*, 1958)

8 *Vitamins A and E*

For convenience these two vitamins are considered together It is not yet possible to say much with certainty how they are related to the diseases under discussion

In normal pregnancy the plasma vitamin A level decreases, while the level of carotene may increase (Lund and Kimble, 1943, McGanity *et al*, 1954 1955) According to Bodansky *et al* (1943), the levels are normal during the first two trimesters of pregnancy and the changes just mentioned occur in the third trimester On the other hand, Cayer *et al* (1947), and the workers in an Amsterdam investigation (Van Bouwdijk Bastiaanse *et al*, 1959) could find no change of the vitamin A level Although the carotene level was normal in Cayer's subjects, it was somewhat decreased in the women of the Amsterdam investigation

According to Lund and Kimble (1943), the levels in a very few cases of toxemia of pregnancy were the same as in healthy subjects Treatment with vitamin A to decrease the toxemia incidence has always been carried out together with other measures For instance, in the People's League of Health survey by Darby *et al* (1953a, b 1955, McGanity *et al*, 1954, 1955), a number of nutrients were given one of them being vitamin A Lund and Kimble (1943) found an indication that fewer complications of pregnancy were seen when the plasma levels were 30 I U % or higher

As to atherosclerosis, Weitzel (1956, 1957) observed a favorable diminution of the fatty deposits in the spontaneous atherosclerotic processes in old hens on administration of vitamin A The effect was much better if vitamin E was given at the same time, but vitamin E alone had almost no effect This combination of vitamins A and E could however produce a hyperlipemia and hypercholesterolemia during the first days of treatment During this time the lipid content of the aorta decreased Though Schettler (1955, 1956) reported favorable effects in man of a combined preparation of vitamins A and E, some complications were encountered later

The vitamin E level increases during pregnancy [e g Kramer 1955, Cervelatti and Massei, 1950 McGanity *et al* 1954, 1955, and the Amsterdam investigation (Van Bouwdijk Bastiaanse *et al* , 1959)] While Cervelatti and Massei (1950) observed also a much lower level of vitamin E in toxemia patients than in normal pregnant controls, Scrimshaw *et al* (1947, 1949) found normal values and Gordon *et al* (1955) and Nitowsky *et al* (1956) noted elevated levels postpartum

The therapeutic effects of this vitamin are complicated For instance, Shute (1953) warned that administration of vitamin E in (pre)eclampsia might be dangerous However, favorable effects which have been ascribed to its vasodilating action were observed in early toxemia Mastboom and Sikkel (1952) could not find a favorable effect in mild cases of toxemia of pregnancy and in a few patients with pre eclampsia, but many of these subjects did not have a genuine toxemia

The toxemia inducing activity of Stamler's cod liver oil diet (1956) disappeared on prior or simultaneous treatment of the animals with α tocopherol The substitution of corn oil for the cod liver oil, which also made the diet nontoxic, provided vitamin E with other factors

In connection with atherosclerosis interest was at first concentrated on the blood levels Like vitamin A, vitamin E is present in all lipid fractions, but especially in the α_2-lipoprotein as are the carotenoids and estrogens (Lewis *et al* , 1954) According to Curcio (1954) the vitamin E levels in old men vary with the cholesterol levels, if the cholesterol level is high, the vitamin E level is also high Chieffi and Kirk (1951) found higher levels in women than in men, except at age over 80 There was a tendency for an increase with age in men, but not in women Generally, young women have lower (β lipoprotein)cholesterol levels than have men of the same age However Vanzetti *et al* (1956) could not find a significant difference between the vitamin E levels in the plasma of healthy young persons and old atherosclerotics The values in diabetics, with their high susceptibility to atherosclerosis, were significantly higher than in both the former groups Vanotti and Gervasoni (1956) found in young healthy subjects a level of 1595 μg vitamin E per cubic centimeter, and in healthy old subjects 2395

μg /cc Higher values were observed in older patients with atherosclerosis In young patients the level was 1600 μg , in the older group 6000 μg /cc Administration of vitamins A and E caused an increase of the plasma cholesterol level, a higher phospholipid level, and higher levels of vitamins A and E The effect was especially marked in the older group Vanotti suggests that vitamins A and E can only inhibit the deposition of fatty material in the vessel walls The results of Schettler and Weitzel with vitamins A and E have already been mentioned

In accordance with the findings of Weitzel (1956) an effect of vitamin E alone on the blood cholesterol level in men could not be found by Keys *et al* (1957c) on doses of 100 mg of α tocopherol However, Wagner (1952) reported an influence of vitamin E doses on the prothrombin time and prothrombin content of the blood Galletti *et al* (1955) similarly found a decreasing effect on the prothrombin time, and also on the recalcification time in patients where these values were already high, tending toward a return to normal However, no effect could be seen in coagulation time, bleeding time, or capillary permeability According to Wagner (1952), histamine has an effect opposite to that of vitamin E, and vitamin E is equally as effective as histaminase in correcting this histamine action

Aftergood *et al* (1956) reported a favorable effect from a fivefold excess of vitamin E on the liver cholesterol values in rats fed lard plus cholesterol Weitzel *et al* (1956) have shown how complicated the relations are between the lipids of blood, liver, and the aorta wall and vitamin A, vitamin E, and lipotropic factors According to Bottiglioni and de Jaca (1955), high doses of vitamin E give a constant decrease in the vitamin A level of the blood, but only slight variations in carotenemia, whereas Beckmann (1955) reported that vitamin E can economize the consumption of vitamin A, and is able, under adequate conditions, to reduce or even normalize the symptoms of vitamin A deficiency without vitamin A being given

A few remarks in connection with the action of hormones may be made here Besides interacting with each other and with the essential unsaturated fatty acids, vitamins A and E influence also hormonal systems

According to Pagliari (1956), vitamin A in high doses diminishes the estrogenic vaginal response in ovariectomized guinea pigs A rather similar conclusion has been reported by Randazzo (1956), who observed a temporary inhibition in the production of estrogens in women An effect like this might not be harmless during some stages of pregnancy Vitamin E in excessive doses gives a prolonged estrone curve in ovariectomized rats receiving an estrogenic preparation (*Vitamin Abstr*, 1956)

The interpretation of all these effects is extremely difficult, and more factors are involved than vitamins A and E It might be suggested that the high cholesterol or, rather, the high α_2-lipoprotein values, of the blood can result in normal blood levels of vitamins A and E or indeed in values

greater than normal Yet the tissue supply of these two vitamins can be too low as a result of a too great inclination to bind to the blood lipids instead of to the normal cell acceptors High doses of these two vitamins may be effective by increasing the tissue supply, but the blood level will be abnormally high and will decrease to normal only if the blood lipid content is also lowered In this way the effects of simultaneously administered substances can be of the utmost importance This is true not only for unsaturated fatty acids administered when vegetable oils are used as the vitamin carrier, but also for the Tweens or similar substances used to obtain water soluble preparations of these vitamins Moreover vitamins A and E are not always synergistic

Just as unavailability for normal metabolism can be suggested for the lipoprotein bound vitamins A and E, so a similar hypothesis can be made for waste products or other "unwanted" substances, cholesterol included, which are bound to lipoproteins or, more generally, bound to the blood lipid fractions This would result in inability to catabolize and remove these products

But altogether it seems probable that there is some direct action of the two vitamins on the lipid system

9 *Vitamin D*

Clear evidence is available of the high susceptibility of vessels to an overdosage of vitamin D According to Ham and Lewis (1934), one single dose of irradiated ergosterol is sufficient to produce more or less intense degeneration and calcification of the muscular media of the vessels within 48 hours Three weeks after feeding large daily doses of ergosterol to very young rats a second lesion appeared a widespread proliferative lesion in the intima If vitamin D administration is stopped, the calcium in the media will disappear, leaving a hyperplastic lesion in the intima

De Langen and Donath (1956), experimenting with rabbits found a marked increase of the cholesterol level following toxic doses of vitamin D Gillman (1957) administered toxic doses of calciferol during 5 consecutive days, and observed severe vascular lesions If these animals were given a high fat diet five to six months after the initial vitamin D experiment the females survived but the males all died within a fortnight Endarterial thickenings and mural thrombi were found Death was ascribed to ' a sudden fall in fibrinolytic capacity of the plasma, perhaps induced by lipemia with subsequent acute, albeit transient, occlusion of the (already) damaged coronary arteries by the deposition of fibrin threads " The females survived perhaps because of their better ability to handle fat and to regulate fibrin precipitation The controls on the original low fat diet survived until killed several months later Their plasma fibrinolytic activity was normal

It would be interesting to know what would have happened if pregnancy

had been induced in the females of this experiment A toxemia of pregnancy might have occurred, as the medium sized arteries in general were reported to be injured and this might have involved the uterine arteries as well Simultaneous changes in heart vessels and aorta, together with changes in uterine vessels, can indeed occur, as has been shown by Rinehart and Greenberg (1956) in pyridoxine deficient monkeys The insufficient blood supply and consequent low supply of oxygen to the placenta and fetus in advanced pregnancy could have been compared with the known facts of this disease in humans Furthermore, Zeek and Assali (1950) actually described an "acute atherosis" (literally stated) of the intima of the decidual vessels which is present in many toxemia cases, and a highly significant correlation between the occurrence of placental infarcts and toxemia of pregnancy Furthermore, they suggested the possibility that an ischemia of the decidua precedes infarction Recently Robertson and Dixon (1958) also reported intimal changes with narrowing of the lumen of the maternal placental blood vessels in pre eclampsia (see Section VIII)

In the experiments of Stamler (1956) experimental toxemia was produced in rats on a diet containing 5% cod liver oil, with about 100 I U per gram of vitamin D This dose is about ten times as high as is used in normal rat rations As cod liver oil contains some highly unsaturated fatty acids which possibly have a toxic effect and may aggravate an essential fatty acid deficiency, one cannot be certain which of these two dietary factors is the more essential for the toxemic effect Sinclair (*1957, 1958a*) *demonstrated* a greater toxicity of vitamin D when the rats were deficient in essential unsaturated fatty acids

The diet which Hoogerwerf (1955a, b) administered to rats to produce experimental atherosclerosis contained 4 75% of a preparation containing 5000 I U of vitamin D_3 per gram (Vigantol) Other authors used similarly high doses, or higher (e g Buckley and Hartroft, 1954), but this may not be necessary in view of the rather small total doses used by the first mentioned authors, namely, one single dose (Ham and Lewis, 1934) or five consecutive doses (Gillman, 1957)

In regard to the possibility of an overdosage of vitamin D, Bedin (1947) reported that vitamin A can prevent toxic symptoms caused by high doses of vitamin D The vitamin A administration, as mentioned in Section II, 8, may be beneficial for this and for other reasons At therapeutic levels, vitamin A increases the good effects of vitamin D

It should be said here that there is a possibility that reactions comparable to those in the animal experiments occur in children The booster doses of vitamin D given a few times a year for the prevention of rickets may be not harmless in this respect Moreover, idiopathic hypercalcemia, which is reported to be relatively frequent in the United Kingdom is possibly not

a direct toxic effect of vitamin D, but there may be an induced hypersensitivity to vitamin D by high doses, and, according to Sinclair (1958a, b), a deficiency of the essential unsaturated fatty acids may play a role also It is reported that cow's milk unfortified with vitamin D can hardly be obtained The fortified milk contains 1400 I U vitamin D per quart, whereas the fortified milk in the United States contains only 400 I U per quart In the United States idiopathic hypercalcemia is relatively uncommon (Bongiovanni *et al* 1957)

10 *Niacin*

Some information is available relating to the role of niacin in atherosclerosis and toxemia of pregnancy Parsons *et al* (1956a, b) described a significant decrease of cholesterol levels in some patients with hypercholesterolemia on administration of 1–4 gm of niacin a day The concentration of the total lipids and the beta alpha lipoprotein ration decreased also, Niacin proved to be the active factor niacinamide in comparable doses did not have this effect Similar results were observed by Galbraith *et al* (1959) in patients with atherosclerosis as well as with healthy young students Discontinuing the niacin resulted immediately in return to the previous levels

Altschul (1956) and Altschul *et al* (1955, 1958) described similar good results following administration of niacin on the blood cholesterol levels in man, and in rabbits with experimental atherosclerosis However the effect seemed to decrease somewhat on prolonged therapy

As to toxemia of pregnancy it was mentioned in the reports of the Vanderbilt Cooperative Study of Maternal and Infant Nutrition (McGanity *et al* 1954, Darby *et al* 1955), that the only significant difference between the diets of the toxemic and the healthy pregnant group was a lower intake of niacin by the toxemic patients This was accompanied by a lower excretion of N' methylnicotinamide in the urine In normal pregnancy there is a higher excretion of this substance than in nonpregnant women (McGanity *et al*, 1954) This may indicate an increased catabolism and therefore a need of niacin Hobson (1948) also found a significantly lower intake (2 mg) of niacin by toxemia patients than by normal controls in Bristol A role of pyridoxine was also suggested but no data were given Further Pai (1956) reported a decreased urinary excretion of niacin, as compared with normal controls in 15 cases with a clinically diagnosed toxemia of pregnancy

11 *Vitamin B_6*

Besides evidence of a relative pyridoxine deficiency in man there is proof that pyridoxine deficiency can produce both atherosclerosis and toxemia of pregnancy in experimental animals Mushett and Emerson (1956) obtained

an experimental atherosclerosis in both monkeys and dogs by administering the pyridoxine antagonist deoxypyridoxine Addition of 2% cholesterol to the diet deficient in B_6 did not cause deposition of lipid material in the arterial lesions (intimal fibrosis) In these experiments dental caries were produced, as well as malalignment of the teeth Rinehart and Greenberg (1956) studied pyridoxine deficiency in the rhesus monkey and demonstrated atherosclerosis, dental caries, and hepatic cirrhosis A suboptimal intake was maintained during several years and the atherosclerotic lesions had a striking resemblance to those occurring in man The important observation was made that similar vascular lesions occurred in the vessels of the uterus, but no investigation was made of the effect of pregnancy in these animals Using transaminase activity values, which are a good measure of relative deficiency of pyridoxine, they demonstrated that maximal values are rarely found in man Glendening *et al* (1955) concluded the same from their experiments in pyridoxine deficient animals and 1% cholesterol added to the diet gave a higher cholesterol level in blood than in control animals receiving vitamin B_6 in the diet, even though the latter ate more cholesterol

Data on the effect of pyridoxine administration alone in patients with atherosclerosis could not be found, but pyridoxine has been used together with other dietary and therapeutic measures (see for instance Schroeder 1955, 1956)

Wachstein (1956) reported an increased excretion of xanthurenic acid in toxemia of pregnancy, indicating a disturbance of tryptophan metabolism The enzyme kynureninase necessary for normal tryptophan metabolism has pyridoxine as coenzyme In normal circumstances the xanthurenic acid is present only in very small amounts Sprince *et al* (1951), Glendening *et al* (1955), Page and Brown (1952), and Page (1956) all found the glutamic aspartic transaminase activity of whole blood in pregnant subjects to be essentially the same as in nonpregnant controls The activity could, however, be increased significantly by a dietary supplement of 10 mg pyridoxine hydrochloride daily As the transaminase activity of fetal blood was found to be twice as great as that of maternal blood, the fetal tissues are considered to contain optimal quantities of pyridoxine, where as adults, both pregnant and nonpregnant, have suboptimal concentrations The fetal supply is presumably at the expense of the maternal tissues Routine administration of pyridoxine did not appreciably decrease the incidence of toxemia, but the metabolic abnormalities were readily corrected by supplementing the prenatal diet with 5–10 mg pyridoxine HCl daily The maternal need is considered to be two or three times higher than normal

Neuweiler (1954) showed an increased need for pyridoxine during pregnancy by a load test with 40 mg pyridoxine orally In the 24 hour urine

4 pyridoxinic acid was measured A tryptophan load test was carried out also by Sprince *et al* (1951), who demonstrated that women with a toxemia of pregnancy excreted more xanthurenic acid than the controls with uncomplicated pregnancy Wachstein and Graffeo (1956) found a lowered incidence of toxemia in a group of 410 pregnant women receiving 10 mg pyridoxine a day The incidence in the control group was 4 4%, whereas the B_6 supplemented group had 1 7% In agreement with others, the authors suggested that in normal pregnancy some insufficiency of B_6 is already present

In 1956 Ross and Pike studied experimental toxemia in vitamin B_6 deficient rats The same changes proved to be present in the serum protein pattern and in the nonprotein nitrogen levels as those seen in toxemic women The effect was similar when the maternal stores were depleted before mating

Day and Dinning (1956) reported on a vitamin E vitamin B_6 interrelationship in the monkey A more severe vitamin B_6 deficiency developed as a result of feeding a diet low in fat and in vitamin E, but this low fat intake may also decrease the severity of the atherosclerotic lesions They showed that pyridoxal phosphate functions as a coenzyme in the desulfuration of cysteine and the decarboxylation of cysteine sulfinic acid An important function of vitamin E is regulation of the sulfur amino acid metabolism and simultaneous deficiency of cysteine and/or vitamin B_6 results in additional pathological signs In this connection, Volovnik (1955) reported that after injection of S^{35} labeled D and L-methionine in pyridoxine deficient white mice the incorporation of S^{35} in the whole body protein and cysteine alone was significantly reduced as compared with nondeficient controls Pyridoxine thus seems to be involved in the synthesis of cysteine by transsulfuration Moreover the conversion of L- to D amino acids (via keto acids) was affected since S^{35} uptake from L and DL-methionine was reduced only 20–27% that from D methionine 44% It is known also that pyridoxine is necessary in the transformation of linoleic acid into arachidonic acid and linolenic acid into hexaenoic acid (Witten and Holman 1952), as well as in various phases of fat metabolism, including oxidation, synthesis especially from protein deposition, and possibly in the transport of unsaturated fatty acids (Tower 1956) Vilter (1956) found that the deficiency symptoms in man can be partly reversed by linoleic acid Pyridoxine inhibits the production of collagen and chondroitin sulfuric acid, as does choline

Important also is the observation of Nelson and Evans (1951) and Nelson *et al* (1951), who proved that it is possible to maintain pregnancy in B_6 deficient rats by administration of estrone and progesterone, whereas this is nearly impossible in nontreated, B_6 deficient animals

Altogether there is no doubt that pyridoxine plays an important role in the etiology of both diseases

According to Schroeder (1955, 1956), the American diet of processed foods may be marginal in respect to pyridoxine content during certain seasons of the year An example of loss of B_6 in food processing and canning is that of a sterilized infant milk formula, the giving of which was followed by severe convulsions which proved to be caused by pyridoxine deficiency (Molony and Parmelee, 1955) Likewise, Leitner (1957) points to the loss of B_6 in canning or processing of food products

12 *Choline*

Data on the role of choline are not numerous, but again some reports have been published for both diseases Its possible role in atherosclerosis is of interest because of its lipotropic properties Wilgram *et al* (1954, 1955) produced coronary and aortic sclerosis in rats by feeding them a low choline diet However, diets effective in producing atheromatous lesions in animals need not be choline deficient (see Section III) Labecki *et al* (1955) administered to their patients a mixture of lipotropic substances containing choline (2 gm of the dihydrogen citrate), methionine (800 mg), and inositol (600 mg) and observed a highly significant depression of the chylomicron levels The decrease in the cholesterol levels was not so clear, but might be present in the myocardial infarction group

The difficulty of this research is exemplified by the results of Sellers and You (1956), who were able to produce coronary lesions in rats with choline deficient and with supplemented diets Rats on the supplemented diet placed in a cold room (1–3°C) had more lesions than those housed at normal temperature, but if made choline deficient the "cold" group showed fewer lesions than the rats housed in normal temperature environment

As to the observed difference in sex susceptibility to atherosclerosis, the findings of Griffith (1940 1941) that young male rats are more susceptible to choline deficiency than females, are important This has been observed by others as well Estrogenic substances have a distinct lipotropic effect According to Schlegel (1949), in women the choline concentration in blood shows cyclical variations completely identical with the shifts in the estrone levels The highest values are present around the 14th–15th, and the lowest around the 26th–28th, day of the cycle

No choline excretion differences between normal pregnant women and toxemia patients could be found by Borglin (1956) Sims (1951) observed the same and reported that methionine excretion is also normal However, the excretion of both substances is much larger in pregnancy than in the nonpregnant state, and blood levels do not change Mischel (1954) measured the choline content of the placentas and found a markedly decreased

content of choline in cases of toxemia The choline level in the blood was likewise lower than in the normal control women Moreover, an experimental toxemia could be produced in animals by giving them a choline-deficient diet This toxemia was strikingly similar to human toxemia

It is very difficult to evaluate these findings, and more than one variable is involved in producing the final result Apparently choline may occupy a key position," but another factor may also have this key position in the long chain of metabolic events concerned Choline is very important, as it is an essential part of the phospholipids and a sufficient supply is necessary to make the production of the required amounts of the various phospholipids possible (see Section IV, 1)

Gyorgi *et al* (1947, 1949) reported that estrogens decrease the need for choline in rats On the other hand, it is suggested that choline metabolism is increased in pregnancy, and choline seems to be necessary in the production of estrogens A decreased oxidation of fatty acids is observed in choline deficiency

13 Cystine and Methionine

Data concerning the role of cystine and methionine are rather scarce Schuck (1950, 1952) reported a lower cystine content of the blood in toxemia of pregnancy The normal value in eight nonpregnant women was 5 3% calculated on pure protein per 100 cc plasma, the corresponding value in ten normal pregnant subjects was 4 5%, and in nineteen subjects with toxemia of pregnancy, 3 6% Normal levels (4 8%) were reached after parturition This is not likely to be caused by hemodilution only since eclamptic symptoms could be produced in animals by giving them cystine deficient diets The fetal blood contains as much as 6 3% Therapy with cystine often causes lowering of the raised blood pressure and lessening of edema, but has little influence on the albuminuria

Simultaneously the methionine levels shift with the cystine but in the opposite direction In nonpregnant women the content was 1 75% in normal pregnancy 2 03%, and in toxemia, as high as 2 34%, after parturition the level decreased again to 2 17%

In concordance with this, Kyank and Franck (1951) reported that the methionine content of the plasma is higher in pregnant than in nonpregnant women with still higher values in toxemia Administration of methionine (4 gm thiomedon) increased the blood level in the healthy subjects, but decreased it in the toxemia patients Bozzo (1952) gave 5 gm of methionine a day and observed a shift in the albumin globulin ratio toward normal values together with an increase of the total protein levels and diminution of edema On the other hand, Ferguson (1956) found no favorable effect on toxemia with a daily vitamin supplement containing 1 gm of

methionine, as well as 20 mg of thiamine HCl, 10 mg of riboflavin, and 100 mg of niacinamide However, the methionine dose was appreciably lower than in the previously mentioned surveys, and most of the patients had a chronic hypertension, and thus not a genuine toxemia Nevertheless a favorable influence on the birth weight and prematurity was shown to be present

Data on atherosclerosis are very scarce Mann and Stare (1954) reported the possibility of predisposing monkeys to atherosclerosis by giving a diet such as to cause a borderline cystine deficiency Labecki *et al* (1955) found an effect on the chylomicronemia in man, with a possible effect on the cholesterol level, on administration of a mixture of the lipotropic factors choline, methionine, and inositol (see Section II, 12) The methionine dose, however, was very much lower than those given to the toxemia groups The problem is complicated further by the Weitzel (1956) report of a pro atherosclerotic effect of methionine (1%) as revealed by the fat content of the aorta in old hens The serum cholesterol level decreased, however Choline and inositol similarly did not give an improvement of the process in these animals (see also Section I, 12)

Further, Jones *et al* (1957) reported that methionine increases the cholesterol level in rats fed a diet containing casein, lard, and percomorph liver oil No influence on the frequency of the lesions was seen, but methionine given together with α tocopherol reduced the hypercholesterolemia to normal levels α Tocopherol alone did not have this effect

One of the pathways for the removal of cholesterol from the blood is by degradation to bile acids (Gordon *et al*, 1957, Lewis, 1958) A deficiency of the sulfur amino acids can be important in limiting the production of taurine, which is required for the formation of taurocholic acid (Filios and Mann, 1954)

14 Copper

Dietary copper does not seem to be important in either disease, except possibly when anemia is present Probably this means that the intake of copper is sufficient under our present circumstances The copper content of the serum, however, is very important in both diseases There is some evidence that the serum protein to which nearly all the copper is bound, the ceruloplasmin, plays the primary role (see below)

15 Magnesium

Magnesium very probably plays an important role in both diseases With the normal "civilized" food products and dietary habits, the daily intake of magnesium is too low (Keeser, 1952) With increasing age the tissue content of magnesium decreases, whereas the calcium content increases If the plasma magnesium level decreases from 2 2 mg % to 1

mg % an increase in the cholesterol level—especially of the esterified fraction—occurs

Magnesium salts, and also magnesium oleate, can reduce high cholesterol levels Seliger (1952) reported a very good effect of magnesium oleate on postprandial hyperlipemia and chylomicronemia The delayed "clearing" seen in older people both in regard to height and duration of the elevated levels can become nearly normal again In agreement with this Bersohn and Oelofse (1957) reported that the Bantu in South Africa had a significantly higher serum magnesium level than a European population The coronary mortality is much lower in the former group than in the latter An inverse relation between magnesium levels and cholesterol levels has been found in these different population groups Moreover, a dramatic improvement has been observed in many cases of coronary disease on administration of magnesium sulfate parenterally Abnormal lipoprotein patterns rapidly reverted to normal

It is true that Brown *et al* (1958) could not find an inverse relation between the magnesium and the cholesterol levels in a group of middle aged American men who had myocardial infarctions and a control group without previous infarction As the intake of magnesium was probably much the same in those two groups and the chance that some of the controls must be considered as future coronary patients might be fairly high this lack of difference is not very surprising Furthermore, if slight differences are expected, it might perhaps be better to estimate the content of intracellular magnesium in the blood cells than the serum levels, since magnesium is mainly concentrated intracellularly

Vitale *et al* (1957) fed rats an atherogenic diet and found fewer atheromatous lesions when magnesium was supplied in an amount of 192 mg %, than in the animals fed 24 mg % The latter dose is the amount adequate for a normal diet The cholate cholesterol diet used produced a magnesium deficiency with low magnesium blood levels, hypercholesterolemia and extensive sudanophilia of the heart and aorta

Biochemical estimations of magnesium in pregnancy are few Hall (1957) reported a slight depression of the magnesium level in pregnancy, without a distinct difference between the healthy and a few toxemic women The lowest values were found between the 34th and 38th week The normal values were as high as 1 87 ± 0 2 meq per liter and individual variations were rather large

The practical effect of high doses administered intravenously is still safe enough for common use in preeclamptic and eclamptic patients Doses of 40–60 gm daily can be given without unwanted effects It is not very probable that this effect could be purely symptomatic (depression of the nervous system and the effect of the hypertonicity of the solution) Moreover, an

methionine, as well as 20 mg of thiamine HCl, 10 mg of riboflavin, and 100 mg of niacinamide However, the methionine dose was appreciably lower than in the previously mentioned surveys, and most of the patients had a chronic hypertension, and thus not a genuine toxemia Nevertheless a favorable influence on the birth weight and prematurity was shown to be present

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acute deficiency or an acute aggravation of a moderate depletion is possible because of the great retention of water and salt occurring i eclampsia and eclampsia In this connection, mention should be m the work of Fitzgerald and Fourman (1956), who tried to induce a nesium deficiency in man with a diet supplying 1 1 meq magnes day during 3 weeks There were no changes in the magnesium blood or in the blood pressure in this relatively short period There was, ho a retention of sodium and chlorine, accompanied by weight gain magnesium is mainly an intracellular mineral it is not surprising t depression of the blood levels does not develop rapidly, and the sa true also for toxemia cases in comparison with healthy controls Ex depletion of magnesium can occur in man as well as in cattle The s toms concern the central nervous system and the peripheral neuromu system, terminating in generalized tonic clonic convulsions and death

In mild cases of toxemia, as well as in coronary sclerosis, therapy wi so called rice water fruit diet may owe part of its good effects to l supply of magnesium and potassium, while the sodium content is This may be as important as the low fat and calorie content of the d

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16 *Sodium and Potassium*

According to the common therapy of toxemia of pregnancy with re tion of salt, one might think that salt plays an important role in the eti of this disease More than once the low toxemia incidence in prim people has been ascribed to their lower salt intake (Van Bouwdijk B aanse, 1954, de Snoo, 1949, Holemans and André 1955) The same n be said concerning some of the observations during World War II, as was rather scarce, regionally, at certain periods (at least in the Ne lands) However, the recent observations of M Robinson (1958) sh stimulate further investigations on this point Robinson reported a tinctly favorable influence of salt prescription in toxemia of pregna Two groups of women were studied, one group was said to take much the other to reduce the salt intake The whole investigation included 2 women The "salt supplemented" group showed a lower incidence of t mia and had less placental infarctions than the "low salt" group Sal large doses (20–30 gm or much more a day) was reported even to cu slight toxemia

Data as to the plasma content of sodium and potassium in pregnancy are not in very good agreement Parviainen et al (1950, 1951) reported a higher sodium and a lower potassium plasma level in toxemia patients than in normal pregnancy In the erythrocytes the differences were even larger Good therapy diminished the differences On the other hand, Dieckman and Pottinger (1956) found a decreased sodium and a normal potassium plasma content in the toxemia cases while in normal pregnancy the sodium level was somewhat elevated as compared with that in nonpregnant women Nordenstrahl (1952) confirmed this but demonstrated in his literature review that the problem is far from simple and that not all data are reliable According to this review sodium and potassium can be lowered in normal pregnancy and increased again when toxemia occurs Tampan et al (1956) noted a retention of sodium and potassium in pre eclampsia and the excretion of it postpartum, they suggested an adrenal cortex hypertrophy due to deficiency of B vitamins Data on sodium and potassium content of the plasma will be influenced by therapy such as salt restriction, moreover, the sodium potassium ratio in cells, and in plasma, as well as in food seems to be very important

In any case eclampsia cannot be a simple sodium intoxication The prescription of a saltless regime in toxemia which has been commonly used until now cannot always prevent the outbreak of eclampsia Furthermore, premature labor and low birth weight of the child, or stillbirth, together with a small placenta, are certainly not rare in chronic cases in spite of salt restriction

As to atherosclerosis and salt there are few observations De Langen (1956) described experiments in which a mixed diet had been given to animals with or without an extra supply of sodium chloride Atheromatous vascular lesions were seen more frequently in the NaCl supplemented group than in the controls The fat and lipid levels of the "NaCl group" also became markedly higher than in the controls The amount of NaCl necessary to obtain these effects varied according to the animal species studied in chickens the effect could be seen with only 1% salt in the diet, while in dogs no salt dose could be found which would produce vascular changes No data on man are known However, there seem to be wide individual differences in the ability to eliminate NaCl This capacity also seems to be influenced by the protein in the diet, the fluid ingested and by hormones These animal experiments are really salt intoxication studies in which severe hypertension is always present The latter is not the case in 'spontaneous' atherosclerosis in humans at least not to so great an extent

Similar loading tests with NaCl have been carried out in rats by Moses et al (1952, 1955) and Meneely et al (1955 1956) with doses from 1 5% to

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13 5 % salt in the food and drinking water *ad libitum*, or with salt drinking water from 1 5 % up to 4 5 % Increasing the potassium c of the diet abolished the nephrosislike syndrome The protective ac potassium and the restoration of the potassium sodium ratio to values, has also been studied by Rahman *et al* (1957) in rats on hi diets They concluded that the lesions seen in the myocardium are c teristic of potassium deficiency and that an adequate supply of pot prevents them

In view of the important role of potassium, it must be remarked diet consisting mainly of vegetable foods provides more potassium does one of animal foods In this respect a toxic effect of sodium appear when the potassium intake is seriously deficient or excret too high

III Dietary Measures Affecting the Diseases in Anima

Many observations in animals have been discussed already in Sect and therefore only a brief survey will be given here

Atherosclerosis can occur spontaneously in various animals, for ins in old hens (Weitzel, 1956) and in the parrot (Cohrs, 1957), and pro in other animals (fed by man?) too The atheroma, easily induced by cholesterol diets in rabbits and chickens cannot be considered comp to the "spontaneous" disease in man Such experiments as that c out by Filhos *et al* (1956), who supplemented purified diets with lesterol, sodium cholate, and thiouracil, should be left out of conside because a low basal metabolic rate was induced However, the rat veloped a blood pattern like that present in the human patient, tog with coronary and aortic lesions, and lesions in the heart valves Be no similar data for toxemia in pregnancy have yet been found, onl results in mammals with a normal metabolic rate will be considered larly, animal experiments in which cholate, cholic acid or other su active agents are used cannot be considered here

Mann and Stare (1954) reported the possibility of predisposing mo to atherosclerosis by giving a diet which caused a borderline *cystin* ficiency In a similar way, Schuck (1950, 1952) found it possible to in symptoms like those of human eclampsia in experimental animals by g them a low cystine diet Hartroft and his group (1952), Best *et al* (1 and Wilgram *et al* (1954) produced atherosclerosis in adult rats with a *choline* diet, with or without vitamin D in high doses Choline more o protected against the lesions caused by vitamin D In this experimen group consuming hydrogenated vegetable oil proved to have a hi incidence of the lesions than the group fed a mixture of beef fat and oil as a fat source Mischel (1954) reported an experimental toxem pregnancy in rats with a choline deficient diet

Wissler *et al* (1954) and Moskowitz *et al* (1956) produced atheromatous lesions in middle aged obese male rats with a diet with an average composition similar to actual diets of certain patients This diet was not choline deficient, but contained lard to an appreciable amount, however, the choline seemed to have an aggravating effect on the lesions The difference in results in this and the foregoing experiments cannot yet be explained It certainly points to the important role of imbalance in the dietary composition and the possible odd effects of relative deficiencies A similar observation was made by Jones *et al* (1957) with methionine which gave a hypercholesterolemia but was without effect on the number of lesions Methionine given together with vitamin E, however, reduced the cholesterol levels to those of the normal controls while vitamin E alone did not have this effect

Others who have experimented with *vitamin D* are Hoogerwerf (1955a, b), de Langen and Donath (1956) and Gillman (1957) and all produced atheromatous lesions in animals Stamler (1956) gave a high cod liver oil (5%) diet to rats supplying an amount of vitamin D of about ten times the normal daily dose but not nearly as high as the dose in the above mentioned experiments Eclampsia in a high percentage of the primigravid rats was observed when this diet was given instead of the normal stock diet at the 13th day of pregnancy Death was preceded by rapid progressive cardiorespiratory failure culminating in violent convulsions Resorption of the fetuses took place in most cases when the diet was given in the first days of pregnancy Multigravid rats were less susceptible than the primigravid Their susceptibility could be increased by giving the diet earlier in pregnancy

The cod liver oil proved to be the critical component of the diet Substitution by corn oil made the diet nontoxic, and crude linoleic acid was approximately as effective The eclamptic disease could be prevented by treating the animals with α tocopherol or lettuce before the toxic diet was given, or by adding these materials to the eclamptogenic diet Apart from a possible toxic effect of the vitamin D it is also possible that the oil itself has a toxic effect It is known that highly unsaturated acids are present which do not have the activity of the essential unsaturated fatty acids, because they have *cis trans* and *trans trans* configurations instead of the active *cis cis* form Even deficiency of the essential unsaturated fatty acids may be promoted The crude linoleic acid which was not quite as active as corn oil may contain about 50% of the inactive *cis trans* configuration(s) The vitamin E content of the corn oil is probably important also

An effect of *niacin* in decreasing the blood cholesterol level was observed by Altschul (1956) Altschul *et al* (1955), and Altschul and Hoffer (1958) in rabbits Schon (1958) found a similar result in the liver cholesterol levels of the rat

Rinehart and Greenberg (1956) induced atherosclerosis in the rhesus

monkey by giving a *pyridoxine* deficient ration The lesions had a striking resemblance to those occurring in man Similar lesions were seen in the uterine vessels, but conditions of pregnant animals were not investigated Ross and Pike (1956) reported that the symptoms of toxemia of pregnancy were induced in rats by pyridoxine depletion Darby *et al* (1955) described an experiment with vitamin B_6 restriction in gravid rats resulting in decreased fertility, increased perinatal mortality, and decreased birth weight The young all died with a striking syndrome of spastic convulsions

The influence of magnesium on experimental atheromatous lesions in the rat was investigated by Vitale *et al* (1957) When an adequate supply of magnesium was given, fewer atheromatous lesions occurred Studies on the effects of NaCl intoxication in rats were carried out by de Langen (1956), Moses *et al* (1952), and Meneeley *et al* (1956)

It should be kept in mind, however, that there is no proof that the disease induced in animals by these various factors, is completely comparable to the disease in man

Summary A low intake of a number of dietary constituents [unsaturated (essential) fatty acids, niacin, pyridoxine, choline, cystine, potassium, and magnesium] seems to be important in the etiology of both diseases The effects of vitamin A, vitamin E, methionine, the potassium sodium ratio, and carbohydrates are less clear, partly because of lack of information in both diseases and partly because the information concerning the role of a particular factor in one of the diseases seems to be somewhat contradictory

No definite effect is exerted by the cholesterol or the protein content of the diet, but a high intake of calories, refined foods, fats, saturated fatty acids, and vitamin D may certainly play a role in the etiology of both diseases

It can no longer be considered as purely accidental that so many nutrients have had to be mentioned, because of all the known interrelations and antagonistic and synergistic actions of these factors However, a full biochemical survey of all these interrelations is beyond the scope of this paper

It should be noted that evidence of a deficiency of certain nutritional factors does not indicate that by providing them at an arbitrary time, when the disease has already been diagnosed clinically, a beneficial effect will always result Irreversible damage may be present already, and temporarily unpleasant or even dangerous effects may occur on supplying certain factors Owing to its rapid course, toxemia has the best chance of giving such complications and these are also less tolerable

When investigating the influence of dietary factors, it should not be forgotten also that food additives, applied for whatever use, may have some influence

IV DEVIATIONS FROM THE NORMAL BLOOD PLASMA LEVELS AN REACTIONS OCCURRING IN THE BLOOD OR BLOOD VESSEL WALLS

Of the blood constituents concerned in the diseases under discussion the most important are discussed below

1 *Serum Lipids*

The whole lipid system in the blood may be considered as a functional unit, with the important task of transporting of fatty acids However, little is known of the exact functions of all constituents The data from studies on cholesterol (free and esterified), phospholipids, α and β lipoproteins[4] and total fat values, in atherosclerosis and toxemia of pregnancy, can often be compared with data on normal subjects and on women with uncomplicated pregnancies There are also some reactions which seem to have a close connection with the lipids, namely blood coagulation, fibrinolysis, and 'clearing "

In atherosclerosis, on the whole, higher levels of cholesterol and phospholipid are present, with a lower phospholipid cholesterol ratio than in healthy subjects (normal value >1) The ratio of β to α lipoproteins is above normal and also the absolute values of α and β lipoproteins are increased The relative amounts of cholesterol in the β lipoprotein fraction are higher Total fat values of the serum are likewise higher than normal (in fasting subjects all serum lipid is bound to proteins)

Individual values of these substances however, do not always give reliable indication as to the presence of atherosclerotic disease This can be accounted for at least partly by the slow development of the complaints and clinical signs, which depend also on the localization, whereas the blood constituents may have been abnormal for a considerable time This is reflected also in the increasing values with increasing age There is no exact information as to normality, and on many occasions levels which initially were considered as within the normal range ought now to be considered as abnormal in view of later information

Since it is impossible to cite all the literature on this subject, only a few citations will be given (see also Symposium on Arteriosclerosis, 1956)

Barr (1956) for instance, showed data on survivors of myocardial infarction in comparison with normal subjects in New York (Table I) Sex

[4] The lipoproteins can be separated by different means paper electrophoresis (main fractions α and β lipoproteins) ultracentrifugation (low and high density fractions) see for instance Fasoli *et al* (1956) and Lindgren and Gofman (1956) or Cohn s *et al* (1950) microfractionating method

influences like those shown here have been reported by many other authors and concern other serum constituents as well. In the above series there is a significant difference between young men and women only in the α lipoprotein levels. The number of subjects, however, is rather small.

Thurnherr *et al.* (1956) gave the averages obtained from Swiss subjects

TABLE I

CHOLESTEROL AND LIPOPROTEIN LEVELS IN SURVIVORS OF MYOCARDIAL INFARCTION COMPARED WITH NORMAL SUBJECTS IN NEW YORK

Age	Number of subjects	Total cholesterol (mg./100 cc.)	Percentage of total cholesterol		
			In α lipoproteins	In β lipoproteins	
Normal men and women					
18–35	44	190	29.3	67.5	
45–65	41	245	23.1	75.2	
Survivors of myocardial infarction					
18–64	33	259	13.6	83.9	
			α Lipoproteins (mg./100 cc.)	β Lipoproteins (mg./100 cc.)	Ratio α : β lipoprotein
Healthy women					
18–35	20	187	533	399	0.75
Healthy men					
18–35	24	197	417	477	1.15
Survivors of myocardial infarction	28	239	467	595	1.25

[a] From Barr (1956).

(Table II) and Antonini and Salvino (1956) reported the data on subjects in Florence (Table III).

Apart from real regional differences in the blood lipid composition, the absolute figures, especially for α and β lipoproteins and their ratio may vary according to the techniques employed.

In general the cholesterol level of the plasma has a tendency to be higher in patients with atherosclerosis. As is well known, the cholesterol value alone cannot give definitive information about the presence of vascular

disease, and the ratio of β to α lipoproteins is also considered very valuable The cholesterol level is often used for comparing groups of subjects and as a criterion for the results of therapy In any case, death from coronary disease tends to be high in populations with levels from 200 mg % up, and low in populations where levels are in the range of 120–180 mg % (Keys et al, 1955, 1956a, b, Bronte Stewart et al, 1955, Bose and De,

TABLE II

CHOLESTEROL AND LIPOPROTEIN LEVELS IN ATHEROSCLEROTIC COMPARED WITH NONATHEROSCLEROTIC SUBJECTS IN SWITZERLAND

Age	Number of subjects	Total cholesterol (mg /100 cc)	Total lipo proteins (mg /100 cc)	α Lipo proteins (mg /100 cc)	β Lipo protein (mg /100 cc)	Ratio β α lipo proteins
Nonatherosclerotic subjects						
<20	7	114	675	195 (29%)	480 (71%)	2 5
>20	14	170	1020	255 (25%)	765 (75%)	3 0
Atherosclerotic patients						
	100	245	1200	270 (22%)	930 (78%)	3 4

From Thurnher et al (1956)

TABLE III

CHOLESTEROL AND LIPOPROTEIN LEVELS AND PROTHROMBIN ACTIVITY COMPARED IN NORMAL AND ATHEROSCLEROTIC SUBJECTS IN FLORENCE

	Normals 40–70 years old	Atherosclerotics
Cholesterol	220 ± 41 5 mg %	223 ± 50 1 mg %
β α lipoprotein ratio	2 65 ± 1 02	4 5 ± 1 12 ($P < 0\ 01$)
Prothrombin activity	100%	118%

From Antonini and Salvino (1956)

1936, Toor et al, 1954, see also Scott et al, 1958 and Corcoran, 1958) Recently Rutstein et al (1958) demonstrated the essential role of cholesterol (as such or as β lipoprotein) in the culture medium for intracellular lipid deposition in tissue cultures of human aortic cells

Elevated levels of cholesterol in normal pregnancy have been reported by many authors Von Studnitz (1955) for instance, showed an increase of cholesterol values from 176 to 276 mg % The latter values may not be pathological, and data on populations where toxemia is rare are needed to

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decide this In rabbits, on the other hand, Baumann and Holly (1926) found that pregnancy causes the cholesterol and blood phosphatides to diminish, and in the dog blood lipids are not affected by pregnancy In any case a physiological increase of the cholesterol level superposed on a high starting value may well result in a level which may be pathological and dangerous It was observed as far back as 1912 that the hypercholesterolemia of pregnancy passes off more quickly in women who nurse their babies (Hermann and Neuman, 1912)

Cholesterol levels in toxemia of pregnancy were studied by Ghose (1953) In 32 patients with pre eclampsia in the third trimester a higher level of total cholesterol was seen than in the normal pregnant controls, and this was mainly due to an increase of the ester fraction Von Studnitz (1955) reports a more pronounced lipemia in toxic pregnancy than in uncomplicated pregnancy One case was reported with a total lipid of 1450 mg /100 cc serum, whereas in normal pregnancy the average is 966 mg /100 cc

It is often assumed that a correlation exists between the raised blood cholesterol level in pregnancy and the increased tendency to the production of gallstones containing cholesterol This might suggest that even in uncomplicated pregnancy ("clinically" uncomplicated), where the cholesterol level is elevated, a physiological borderline may already have been surpassed in a number of cases As to toxemia of pregnancy, with its still higher cholesterol levels, this probability is still greater A strong argument is found, moreover, in the "acute atherosis" of the decidual vessels, described by Zeek and Assali (1950) in toxemic cases, and in the intimal changes in the maternal placental blood vessels reported by Robertson and Dixon (1958) in pre eclampsia

However, changes in other blood constituents must also be considered For instance, the phospholipids are very important factors These compounds seem to have the special task of holding the cholesterol (esters) and other lipids of the blood in "solution" in cooperation with the plasma proteins The synthesis of the phospholipids occurs mainly in the liver and, according to Reiser (1955), in the intestinal wall, but also in other tissues, including the aorta wall Choline is necessary as it is an essential part of the lecithin molecule This would at least indicate that its simultaneous intake with dietary fat is important Indeed, the lipotropic effect of choline is considered to be due to its share in the synthesis of phospholipids (*Nutrition Revs*, 1957) There are, however, other phospholipids, the most important of which seem to be those in which ethanolamine replaces choline in the molecule (cephalin) Poole and Robinson (1956) demonstrated that this phospholipid has an accelerating effect on blood clotting by increasing the formation of thrombin in the coagulation system This is in contrast to the normal choline containing lecithin, which has no such activity Phospholipid synthesis is stimulated by the absorption of fat, in general

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total lipid phospholipid ratio of 966 398 or 100 41 2 in healthy pregnant women and 664 242 or 100 36 5 in normal nonpregnant subjects Kaplan *et al* (1957), however, found a normal ratio of only 700 210 or 100 30 (United States)

The above findings seem to be in contrast with the data of Oliver and Boyd (1953, 1955a, b), who studied twelve young healthy pregnant women in Edinburgh As pregnancy advanced a gradual rise in cholesterol level was seen together with a decrease of the phospholipid cholesterol ratio and an increase of the concentration of cholesterol in the β lipoprotein fraction By the thirty second week the levels and the distribution of the lipids and lipoproteins in each woman resembled those seen in cases of coronary disease However, the pattern returned to normal about the fifth month postpartum Oliver and Boyd (1955a) gave a preliminary report on the histories of 500 postmenopausal women with coronary disease and 500 healthy women of comparable age The number of pregnancies in the former group proved to be significantly greater than in the healthy group In studying the influence of the menstrual cycle, on the other hand, they found that the effect on the cholesterol, cholesterol phospholipid ratio, and lipoproteins was the reverse of that in the pregnant women At about the time of ovulation, corresponding to a peak in the secretion of estrogenic hormone, the cholesterol level was lower and both the α to β lipoprotein ratio and the phospholipid cholesterol ratios were higher These effects correspond with the results of estrogen therapy in patients with coronary sclerosis, who show marked changes in the various blood levels in the "favorable" direction

Russ *et al* (1954) (United States), studying the relations in twenty seven healthy pregnant women could also not find these "normalizing" trends of the estrogens in the blood lipid pattern in the mothers, but in the children (cord blood) the lipid pattern proved to be such as would correspond to estrogen administration A similar lipid pattern in infants has been reported by Rafstedt (1955)

From these data one might suggest that these women were not completely healthy, at least insofar as they could not respond without any pathological sign to a higher demand on the system regulating the blood lipids and perhaps other systems These subclinical signs might still be considered as a subclinical toxemia It is tempting to suggest that a lack of estrogens may be responsible for this In any case, this view is in agreement with the findings of Smith and Smith (1937, 1939, 1940, 1946, 1949), who reported that the estrogen content of the blood is lowered in toxemia of pregnancy, while the level of the gonadotropins was raised They demonstrated these changes 5–6 weeks prior to the clinical evidence of toxemia This is in agreement with Anker (1956), who found a very low

estrogen content in the urine in a case of severe pre eclampsia (±1 mg per liter instead of a normal value ranging between 6 6 and 21 4 mg per liter)

Moreover, Gemitz and Schild (1955), in Germany, found lipoprotein changes in 86 normal pregnant women, which are opposite to those reported by Oliver and Boyd, and which thus are in agreement with the cyclical effects in nonpregnant women and the effects of estrogen therapy The α_1 lipoprotein increased by 6% on an average, the β lipoprotein did not change, and the "trail" (mostly neutral fats) decreased by 8% Only in the first weeks of pregnancy were the effects sometimes reversed, and this could then perhaps be ascribed to a transient delay in estrogen production

As there is a phospholipid cholesterol ratio of about 1 1 or less in atherosclerotic patients and a somewhat higher ratio in healthy subjects, it seems that pregnancy shifts the ratio in the "favorable" direction, perhaps "normalizing" it The optimal ratio seems to be fairly high—about 1 4 If now a toxemia develops the ratio will probably decrease, but will still be higher than in the average "healthy" subject, for a certain period of time The same argument can be used to explain the other blood constituents

If the hypothesis can be accepted for a moment that a whole range of amounts of estrogen secretion is present in a group of women it is conceivable that there are many women who never will show any clinical evidence of toxemia, but who may have blood changes that predict the onset of the disease but in whom the outburst of the clinical signs is canceled by delivery

The actual frequency of toxemia of pregnancy in relation to age, parity twin pregnancy etc could be explained, perhaps, by considering the effect of the estrogens on the growth of the necessary new blood vessels in the uterus wall and in the placenta The whole problem is reduced in this way to that of hypoxia which is generally accepted as the real "cause" of all troubles Of course, insufficient vessel supply is not the only possible cause of hypoxia Other causes may include pathological changes—for instance atherosclerosis—in the (normal amount of) vessels spasms possibly also mechanical factors, and congenital abnormalities

The probable presence of a wide range of gradations of toxemia which can be seen in terms of blood and blood vessel supply as well is indicated by the observations of Mastboom (1948) and Theobald (1958) If a toxemia develops early in pregnancy, the patient has a fair chance of developing toxemia again in a second pregnancy but it usually occurs somewhat later If the toxemia occurs at the same time as in the first pregnancy then according to Theobald this patient will always develop toxemia during pregnancy unless sympathectomy or something of the kind is carried out

Those with the first signs of a toxemia at the 39th or 40th week of their first pregnancy are not likely to develop toxemia in the next pregnancy

If the role of the estrogens (progesterone has no distinct influence on the blood lipid pattern) is really so important in this respect, it will be clear that further determinations of the lipid pattern in clinical cases of toxemia could be very helpful The changes in the lipid pattern, or its tendency to shift in a particular direction, might resemble what occurs in cases of coronary disease or atherosclerosis The hyperlipemia and hypercholesterolemia were already mentioned above Von Studnitz (1955) described the electrophoresis pattern of one case of toxemia of pregnancy A much higher amount of β lipoprotein seems to be present in the toxemic pregnant, than in the normal pregnant woman, together with a much higher β α ratio, gradually normalizing during the first 6 postpartum weeks The same effect has been described by Loeffler and Wunderly (1953) In the normal pregnancies described by Von Studnitz the α lipoprotein level showed an increase from 224 to 326 mg % with corresponding levels of the β lipoprotein from 375 to 597 mg % (calculated ratios are β α from 1 7 to 1 8) α Lipoprotein fell from 37% of total fat to 33%, β lipoprotein increased from 52% to 62% of total fat

A comparison of the incidence of toxemia measured according to the same criteria in Istanbul (Arnakı, 1956), Malmo (Von Studnitz, 1955), Edinburgh (Oliver and Boyd, 1953, 1955a, b), the United States (Peters *et al*, 1951), and India (Chatterjee and Gosh, 1940) would certainly be of value

It should be noted that normal pregnancy (Russ *et al*, 1954, Von Studnitz, 1955) as well as an injection of heparin in nonpregnant individuals (Kuo *et al*, 1956, Laurell, 1955, Dangerfield and Smith, 1955) increase the migration rate of the lipoproteins

Considering once more the phospholipids, it should be borne in mind that the need of the fetus for these "building stones" in the last months of pregnancy is very high and the turnover in the placenta is enormous Phospholipid supply in toxemia of pregnancy will probably be too low This might be concluded from the lower weight of placenta and child But how this would influence the maternal blood level cannot be predicted with certainty Blood phospholipid levels in clinically established toxemia have not yet been found in the literature

According to Popjak and Beeckmans (1950), the placenta takes more phospholipids from the maternal circulation than does the liver Boyd and Wilson (1935) estimated that the fetus takes up roughly 30 gm of phospholipids a day from the umbilical vein blood at the time of birth Apart from this supply of phospholipids from the placenta—after disintegration and rebuilding, because phospholipids cannot pass across the placenta—

the fetus synthesizes phospholipids in its liver (Popják and Beekmans, 1950, Arnaki, 1956) and in other tissues According to Arnaki the amount taken up from the umbilical vein blood would provide approximately the amount of calories necessary for the basal metabolism of the child

In this connection it seems curious that choline excretion is very much higher in pregnant than in nonpregnant women (Sims, 1951) Borglin (1956) reported a slightly increased excretion during the first months of pregnancy, a strong increase after the fifth month, and between five and ten times the normal level at the time of delivery The levels were normal again within 1 week postpartum In toxemia of pregnancy no different excretion pattern was seen by Borglin (1956), but on the other hand Mischel (1954) reported that the choline levels of the blood and placenta are lower in women with toxemia of pregnancy than in women with an uncomplicated pregnancy Moreover Mischel could induce in animals an experimental toxemia by giving them a choline deficient diet

It would be of interest to repeat the work of Schlegel (1949), who found cyclic variations in the blood choline content paralleling the changes in estrogen content

Sims (1951) and Borglin (1956) suggest that a deficient choline intake cannot be a primary cause of toxemia Obviously it need not be for the impaired production of phospholipids may be due to a deficiency of other factors, such as lack of the essential fatty acids, or of pyridoxine, which is necessary for transformations to the higher unsaturated fatty acids such as are present in the "normal biological" phospholipids Halden and Prokop (1957), citing Grun and Limpacher (1923), points once more to the fact that only the phospholipids with unsaturated fatty acids have biological activity, indeed, analysis of blood and other phospholipids shows the presence of highly unsaturated fatty acids (see e g Wittcoff, 1951)

Choline has also been mentioned in connection with atherosclerosis problems Hartroft *et al* (1952) Best *et al* (1955), and Wilgram *et al* (1954, 1955) reported atheromatous vascular lesions in the coronary arteries and sclerosis of the aorta in choline deficient rats, together with kidney lesions The sclerosis resembled the Monckeberg type in man Wissler *et al* (1954) and Page and Brown (1952) by means of a choline supplemented diet induced vascular lesions in animals which even more closely resembled the lesions seen in man The data on atherosclerosis agree with those of pregnancy in that it is obviously not the choline which is the limiting factor in all cases However such cases might occur

Considering all the foregoing facts it is indeed clear that the administration of extra choline does not promise good results in all cases either in atherosclerosis or in pregnancy It is clear also that much information is needed from population groups where toxemia of pregnancy does not occur

and where vascular disease also is infrequent Corresponding values on animals can be of great help, too

2 *Blood Coagulability and Fibrinolysis*

In hypercholesterolemia or hyperlipemia in general, such as occurs in atherosclerosis, the *clotting time* of the blood is shortened Macfarlane *et al* (1941) and D S Robinson *et al* (1956) showed relations to exist between the chylomicrons in the blood and the clotting time Maclagan and Billimoria (1956) demonstrated that the clotting time was shortened more by the consumption of butter than by that of margarine, yet the two fats produced a plasma turbidity of the same order As noted above, the nature of the phospholipids present in the food or those produced during the absorption process very probably play a role Ethanolamine phosphatide, which is present in butter and other dairy products and which can be found in the chylomicrons in the plasma, is a potent activator for the formation of plasma thrombin (Poole *et al*, 1955, Poole and Robinson, 1956 D S Robinson *et al*, 1955, 1956, Robinson and Poole (1956), O'Brien, 1956a, b, 1957) and thus has strong coagulation inducing properties

Pilkington (1957) demonstrated a similar relation between the ingestion of cream and of olive oil The saturated fatty acids in cream accelerated the clotting process while the unsaturated (mostly oleic) acids of the olive oil had no influence on the clotting time However, the effects of fatty meals on the blood clotting times vary according to the methods used, i e to the specific parts of the chain of reactions resulting in blood clotting which are measured Moreover results are not related in a simple way either to chylomicronemia or to cholesterol depressing or elevating effects (see Keys *et al*, 1957a–d, Mersky and Nossel, 1957, Kingsbury and Morgan, 1957, Fullerton, 1956)

Greig and Runde (1957) showed that butter fat and egg yolk inhibit fibrinolysis, whereas, after consumption of vegetable oils, the fibrinolysis usually rose above the fasting level, if it was low initially This effect was unrelated to the degree of saturation of the fatty acids β Lipoprotein, the level of which is high in atherosclerosis, inhibits fibrinolysis (see also Barnard and Barnard, 1956, and Gillman *et al*, 1957) Moreover fibrinolysis is influenced by sex hormones estrogens increase the fibrinolytic activity (Gillman *et al*, 1957)

The clotting of blood itself also produces an increased fibrinolytic activity This has been reported also after intravascular clot on abruptio placentae (Schneider and Engstrom, 1954, Schneider, 1955, Larkin and Phillipp, 1957) Recently Biezenski and Moore (1958) reported on fibrinolysis in 500 cases, over 90% of whom had a clinically normal pregnancy The incidence of lysis fell progressively after the 15th week of pregnancy,

reaching the lowest value (about 10%) during the last 10 weeks, and the first two stages of labor Three hours postpartum the value was already about 75%, and at 24 hours postpartum the normal nonpregnant incidence of 90% was reached As lipemia inhibits fibrinolysis (Greig and Runde, 1957) and estrogens increase it (Gillman *et al* 1957), there are at least two factors acting antagonistically If there is a too high lipemia and a too low estrogen production, as is suggested now to be present in toxemia of pregnancy, fibrinolysis will be low and might then be insufficient to prevent the formation of thrombi, or insufficient for the lysis of fibrin already deposited This might concern the vessels supplying the placenta, but also other maternal vessels The events taking place in the latter might be compared with the events occurring in atherosclerosis This accords well with the findings of Oliver and Boyd (1955a) that postmenopausal women with coronary heart disease had had more pregnancies than the healthy controls Moreover a more direct description of such events has been given by Schneider and Engstrom (1954) and Schneider (1955), who point to the release of thromboplastin on autoextraction of a retroplacental hematoma This can give such a strong inclination to blood clotting that fibrin deposits are caused in the pulmonary circulation in such amounts that an acute cor pulmonale can occur (see Section VIII)

Lack of physical activity has been reported to result in reduced clotting time because of the longer lasting hyperlipemia and inhibition of fibrinolysis Stress increases fibrinolytic activity Hyperlipemia is constantly present in atherosclerotic patients and their tendency to thrombosis is known to be higher than normal (McDonald and Edgill 1957) Duguid (1956) and Duguid and Robertson (1957) even suggested that organization of mural thrombi produced the atheromatous plaques, and Antonini and Salvino (1956) reported a prothrombin activity of 118% in atherosclerotic patients in comparison with healthy controls

A reduced clotting time in toxemia of pregnancy has been described by various authors Bajusz (1955) demonstrated a hyperthrombinemia in experimental toxemia in animals and ascribed it to a too low level of heparin Chicco (1950–1951) showed a shorter clotting time in patients with a toxemia of pregnancy, and normal values in controls with an uncomplicated pregnancy Perrini (1950) measured the coagulation time the blood prothrombin, and the thromboplastic activity and compared this with the values of 70 normal nonpregnant women His data are tabulated in percentages of normal (Table IV)

The coagulation time and prothrombin values decreased as the severity of the toxemia increased Heparin values would be of interest but these were not given Return to normal was slow in the eclampsia cases, indicating more chance of vascular accidents postpartum Placental infarcts are in

deed often present in toxemia cases, and thrombosis postpartum is also more often seen than in uncomplicated cases Holmer (1947), who reported a decreased incidence in the Netherlands of toxemia of pregnancy during World War II, also remarked that placental infarctions, as well as calcium incrustations, were nearly *completely absent, yet they* are frequently seen at any other time

3 Clearing Factor, Heparin, Mucopolysaccharides

"Clearing" of the lipemic plasma is produced by the clearing factor, a complex thermolabile system activated and stabilized by heparin or related substances Doses of heparin given endogenously as well as intravenously are effective In alimentary lipemic plasma and in other protein containing

TABLE IV

Coagulation Time Blood Prothrombin and Thromboplastic Activity in Pregnant Subjects Compared with Normal Controls

	Normal in third trimester (70 subjects)	Moderate toxemia (23 subjects)	Eclampsia (15 subjects)
Coagulation time	Slightly decreased	Further decreased	Greatly decreased extreme coagulability
Blood prothrombin	109% (7th month) 118% (at term)	Decreased	Nearly always below 70%
Thromboplastic activity of plasma	88 4% (at term)	Max 156 5%	Max 401% very high

[a] *From Perrini (1950)*

neutral fat emulsions, heparin causes a rapid "clearing" (first observed by Hahn, 1943) in which the neutral fat carrying particles, the chylomicrons and the lighter molecules of the β lipoprotein group (Gofman's S_f 20–400 fraction) decrease or totally disappear *while the high-density* lipoproteins increase (Lindgren and Gofman, 1956, Graham *et al*, 1951) According to Kuo *et al* (1956) and others, heparin also causes a marked increase in the electrophoretic mobility of both α and β lipoproteins, but no conversion of β- into α lipoproteins

Only the protein bound triglycerides are the substrate, the enzyme thus being a lipoprotein lipase (Korn, 1954 1955) As a result of the splitting the neutral fats are converted into glycerol and fatty acids, which latter are held in solution by binding six molecules of fatty acid to one of albumin (Gordon *et al*, 1953, Nikkilä and Haahti, 1954, Robinson *et al* 1953, 1954) and also by binding to β lipoprotein (Nikkila and Haahti, 1954)



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neutral fat emulsions, heparin causes a rapid "clearing" (first ob Hahn, 1943) in which the neutral fat-carrying particles, the chyl and the lighter molecules of the β-lipoprotein group (Gofman's fraction) decrease or totally disappear while the high-density lip increase (Lindgren and Gofman, 1956, Graham *et al*, 1951) Acc Kuo *et al* (1956) and others, heparin also causes a marked incre electrophoretic mobility of both α and β lipoproteins, but no c of β- into α-lipoproteins

Only the protein bound triglycerides are the substrate, the enz being a lipoprotein lipase (Korn, 1954, 1955) As a result of the the neutral fats are converted into glycerol and fatty acids, whi are held in solution by binding six molecules of fatty acid to one of (Gordon *et al*, 1953, Nikkilä and Haahti 1954, Robinson *et al* 19 and also by binding to β lipoprotein (Nikkilä and Haahti, 1954)

judge by the biochemical findings in such cases Other observations con cerning myocardial infarction are those of Pomerance (1958), who found considerable excess of mast cells in coronary arteries at the sites of a fres thrombus, while some local increase over atheromata was also present Dat given by Sundberg (1955) and in the literature support this Furthermore a higher mast cell count was found in the coronary arteries in patients wit diseases in which there is a tendency to thrombosis These local effects and the absence of age influences in these cases, are not necessarily i contradiction with the findings of Hellstrom and Holmgren The mast cell can migrate by ameboid movements and might perhaps do so toward th sites of local need i e the vessels As there is an increase of atheromatou lesions with increasing age whether or not succeeded by clot formation this might explain the decreasing counts in the skin and heart muscle tis sue This effect might be even greater if indeed the production of new hepa rin is impaired, as is suggested by the data already mentioned in this sec tion This idea could be supported by the fact that Pomerance (1958) di not observe higher counts when the thrombus was not of local origin Furthermore, Poller (1958) demonstrated that clot formation increases th level of factor VII, and that the release of factor VII activity would b greatest at the site of the thrombus Since factor VII powerfully antago nizes the anticoagulant effect of heparin, the increased mast cell coun might be a response to the raised factor VII level which will neutralize th heparin circulating in the blood

Data concerning heparin and mucopolysaccharides in toxemia of preg nancy are extremely rare Mischel and Mischel (1955) reported a decrease total lipase activity of the blood in toxemia cases Nothing is said about th clearing factor or heparin but on the other hand they reported that th atoxyl fast and quinine fast lipase fractions were increased, whereas thes fractions are not mentioned in atherosclerosis research Mischel and Mische found normal values of the lipase activity in other complications of preg nancy, for instance, in hyperemesis Bajusz (1955) suggested a low heparin content of the blood as the cause of the higher tendency to blood clotting in toxemia of pregnancy Kaku (1953) described isolation of a polysac charide with antigenic properties from the placentas of women with a toxemia of pregnancy Injection of this polysaccharide into pregnant ani mals induced the production of antibodies as in the toxemia patient and gave rise to symptoms of real toxemia This fact is important in connection with Antonini and Salvino's data on the production of qualitatively ab normal polysaccharides with antigenic properties in atherosclerosis

Further evidence for a disturbed mucopolysaccharide metabolism is re ported by Fekete (1958) In edema of pregnancy mucopolysaccharides which have the tendency to bind water and salt, are accumulated This

and Salvino, 1956) In concordance with this the number of mast cells (producing the endogenous heparin) in the connective tissue is diminished in atherosclerotic subjects Hellstrom and Holmgren demonstrated as far back as 1947 that the number of mast cells per tissue unit, recorded in 100 μ slices of heart and skin, was greater in females 0–39 years of age than in males of the same age range In both the skin and the heart, the counts decreased significantly with increasing age In the skin the differences between men and women disappeared, whereas in the heart older women had lower counts than men of the same age Sundberg (1955) reported very similar data As the α to β lipoprotein ratio, cholesterol levels, and other levels show variations in the same sense as heparin levels and mast cell numbers, it can readily be accepted that hormones must play a role in this whole complex of events

Antonini and Salvino (1956), citing Engelberg (1953) and others, point again to the fact that an increase of heparin occurs during the menstrual cycle mainly in the folliculinic period Estrogens are capable of increasing the release of heparinoid substances from the tissues, simultaneously with an increase in the blood concentration of the glucosamine and nonglucosaminic polysaccharides They also normalize hypercholesterolemia and alter lipoprotein distribution, whereas androgens aggravate the symptoms (Barr, 1956, Boyle, 1956) A further argument for a relation between heparin and estrogens is found in the data on the estrogen excretion described by Kirk (1951) (see Section VI) A similar sex and age distribution pattern is shown as that in the mast cell counts reported by Hellstrom and Holmgren (1947)

According to Stary *et al* (1951b), the aminopolysaccharides containing glucosamine—in contradistinction to other polysaccharides which contain only the hexoses mannose and galactose—participate in the formation of gonadotropins, prothrombin, heparin, and blood group substances, and constitute prosthetic groups of several compound proteins The level of the protein bound polysaccharides is increased in a number of diseases, including pneumonia In normal pregnancy the albumin bound fraction is also significantly increased In diabetics, however, the polysaccharide content of the serum proteins is increased also, but the surplus is more or less equally divided between the albumin and globulin-bound fractions The same pattern could be found in alloxan diabetic animals In old age, where more or less atherosclerosis is commonly present, the blood polysaccharides increase in quantity and they develop specific immune reactions (Antonini and Salvino 1956)

Linko and Waris (1955) report that mucoprotein levels may rise considerably after clinical myocardial infarction and that the values remain above normal for at least 3 weeks This finding may have significance also in small subclinical infarctions, whether in the heart or in the placenta, to

nology varies somewhat, especially as to pre eclampsia, judgment may b difficult on some occasions

The alterations in the serum proteins seen in cases of atherosclerosis ar qualitatively similar to those seen in toxemia of pregnancy Kheim *et a* (1953) reported electrophoretic studies of serum proteins in 30 athero sclerotic men and 30 women and also in 20 men with coronary disease A hyper beta globulinemia was present and was accompanied by hypo albuminemia in almost all cases 'The deviations from the normal averag were more striking the closer was the occurrence of the coronary occlusion Russ *et al* (1951) and Barr *et al* (1951) reported a lower albumin conten of the serum of the older age groups and in survivors of myocardial infrac tion than in the younger age groups Voigt *et al* (1956) gave the followin data on this subject in patients with vascular disease of the fundus vessels *total protein* normal 7 21%, atherosclerosis 6 84% (mean calculated fron the data of the four groups of patients), *albumin*, normal, 58% of tota protein, atherosclerosis, 56 4% of total protein, the globulin values thu being 42% and 43 6% of total protein The hypoalbuminemia has beer described also by others

The deviations from the normal values are generally rather small in atherosclerosis in comparison with those in toxemia of pregnancy Thi might be explained by the fact that the atherosclerotic disease is chroni in comparison with the relatively acute course of toxemia in pregnancy I atherosclerosis there is no urgent need and no possibility for a large an acute compensation such as is necessary to keep the blood supply in th pregnant uterus at normal levels Raising the blood pressure and increasin the blood volume takes less time than producing the proteins correspondin to this increase in blood volume

5 *Ceruloplasmin*

Ceruloplasmin is a special protein of the blood plasma mentioned fre quently both in connection with atherosclerosis and pregnancy This blue copper containing protein was isolated first by Holmberg and Laurell i 1948 It is an alpha globulin with a copper content of 0 32–0 35% with 8 atoms of copper per molecule and molecular weight of about 151 00 (Pedersen 1945) Ceruloplasmin has an oxidase activity with numerou substrates, e g ascorbic acid, *p* phenylenediamine, benzidine etc (Holm berg and Laurell, 1947, 1951a b c) There are two fractions of plasma cop per in normal subjects, which have been referred to as the ' direct reacting' fraction (0–17% of the total) and the "indirect reacting" fraction indicat ing a direct and an indirect action respectively with the copper colorimetri reagent sodium diethyldithiocarbamate The direct fraction is bound t serum albumin (Bearn and Kunkel, 1954) and seems to be actively con

property is lost on exposure to hyaluronidase It can be suggested, therefore, that in both diseases mucopolysaccharide metabolism is inhibited or is led partly to abnormal end products instead of yielding the heparin or related substances which are necessary The estrogens seem to have, primarily or secondarily, an important role in the normal course of events

4 Serum Proteins

As will be understood, the levels as well as the ratios of the serum proteins are of interest It has been known for a very long time, and agreed to by many authors, that normal pregnant women show a slight decrease of the total plasma protein content, a decrease of serum albumin, and an increase of the serum alpha and beta globulin, while gamma globulin remains unchanged or may diminish Friedberg (1951) found these changes more marked in toxemia of pregnancy Glatthaar *et al* (1951), however, could not find a significant difference between normal pregnant and toxemic women The albumin level decreased to 50 % of the total protein, whereas values in normal nonpregnant women were 65–70 % of total protein Stary *et al* (1951a) reported a decrease of total protein from 7 8% to 6 6% (9th month) with an albumin level of 5 0% and 3 7%, respectively, while the globulin levels were 2 4% and 2 7% in normal pregnancy (9th month), toxemia data are not given Ross and Pike (1956) demonstrated similar changes in the serum protein pattern in normal pregnant rats In experimental toxemia induced by a B_6 deficiency in these animals, the decrease of serum protein was larger, and the increase of the plasma globulin greater, effects which they also observed in patients with a genuine toxemia

In cases of very serious toxemia (pre eclampsia or eclampsia) the globulin content has a tendency to decrease again in women as well as in B_6 deficient toxemic rats This decrease is understandable if one considers the hydremia and/or edema production together with proteinuria, in which much globulin and albumin are lost and all plasma proteins except fibrinogen can be found in the urine (Parviainen *et al* , 1951) At first, loss of protein is met by production of new serum protein, particularly globulin, which appears easier than albumin to synthesize in the body This compensatory mechanism, however, cannot meet all demands the more so because the globulin has much less oncotic activity than albumin and thus favors the production of edema and hypoproteinemia

It should be kept in mind that toxemia data can be used for comparison with data on atherosclerosis only in those cases where kidney disturbances are not serious enough to alter the original pathological relations in the blood and the urinary excretion pattern This means that data on severe cases of pre eclampsia and eclampsia often cannot be used But as termi

(1955) for instance, reported an average total serum copper of 192 μg /100 cc in patients with a chronic infection

Values in toxemia of pregnancy were described by Thompson and Watson (1949) The serum copper level in 12 cases of pre eclampsia (blood pressure over 160/90 together with either albuminuria or edema after the 30th week of pregnancy) showed a small but statistically significant increase over the usual elevation observed in pregnancy, but there seemed to be no correlation between the severity of toxemia and the height of the serum copper level The mean value in this series was 279 μg /100 cc , and in 42 cases of uncomplicated pregnancy (37–40 weeks) a mean value of 243 μg /100 cc was found But a ceruloplasmin loss by kidney lesions is possible similar to that which occurs in nephrosis and this might lower previous high levels somewhat Ninety per cent of the copper was bound in the globulin fraction

The serum copper level is elevated after myocardial infarction (Russ and Raymunt, 1956) Vallee (1952) and Adelstein *et al* (1956) demonstrated that the change ordinarily starts on the second or third day after the infarction, continues to a maximum on the fifth to the eleventh day and gradually decreases to normal in several weeks The extent of the copper elevation during this period is greater than can be accounted for by the cardiac copper content freed by tissue necrosis The maximum levels reported by these authors were between 151 and 227 μg /100 cc

There is no definite correlation with the erythrocyte sedimentation rate but as the latter is dependent on changes in globulin as well as in fibrinogen content, a correlation with the total globulin content is not impossible (in any case ceruloplasmin belongs with the globulins) This also would not be in contradiction with the hypercupremia seen in so many apparently unrelated diseases Changes in globulin content are at least as nonspecific The globulin content is higher in pregnant than in nonpregnant women, and in toxemia of pregnancy this effect is even stronger In the latter the high ceruloplasmin levels may also be due to placental infarctions, which (see Section VIII) can be demonstrated in nearly all toxemia patients with certainty whereas these are absent, or less marked, in the placentas of clinically normal pregnant women

Copper and ceruloplasmin levels in subjects with known atherosclerosis or coronary heart disease without infarction have not yet been compared with those of normal subjects This will be difficult because the range of values considered as normal is very wide The values of Russ and Raymunt (1956) for the xanthomatosis patients with their vascular lesions that are very probably present suggest that levels in atherosclerosis may be slightly above normal 152–175 μg /cc (slightly elevated globulin level and perhaps microthrombotic processes or infarctions) The levels reported by

cerned in copper transport The indirect reacting fraction corresponds to the ceruloplasmin bound copper and is not concerned in copper transport

Hypercupremia and hyperceruloplasminemia are present in pregnancy and after myocardial infarcton, but also in acute leukemia and infections Very low values are seen in Wilson's disease and in nephrosis (20–80 μg %) Normal copper values range from about 80 to 140 μg per 100 cc (Russ *et al*, 1954, Russ and Raymunt, 1956, Adelstein *et al*, 1956, Lahey *et al*, 1953a, b, Markowitz *et al*, 1955) Lahey *et al* (1953a) demonstrated a significant sex difference, the average value in 23 normal females being 116 ± 16 (range 84–143) μg per 100 cc, and in 40 healthy males, 105 ± 16 (range 68–134) μg per 100 cc

In normal pregnancy the serum copper level begins to rise gradually at

TABLE V

COPPER AND CERULOPLASMIN LEVELS AND OXIDASE ACTIVITY COMPARED IN NORMAL SUBJECTS AND IN PREGNANCY

	Normal[b] (♂ and ♀)	Pregnant[b] (third trimester)
Total serum copper	108 ± 9 μg /100 cc	257 ± 38 μg /100 cc
Direct reacting copper	5 ± 6 μg /100 cc	29 ± 13 μg /100 cc
Indirect reacting copper	103 ± 11 μg /100 cc	228 ± 35 μg /100 cc
Ceruloplasmin	34 ± 4 mg /100 cc	84 ± 15 mg /100 cc
Oxidase activity	3 9 ± 0 7 μmoles O_2/ml /hr	9 2 ± 2 2 μmoles O_2/ml /hr

From Markowitz *et al* (1955)
[b] Average of ten subjects

about the 8th week, reaching a maximum between the 32nd and 36th weeks, which remains until delivery (Krebs, 1928) The level is generally about twice normal Return to normal occurs in the 5th to 10th week postpartum Fay *et al* (1949) reported normal levels of 122 7 ± 10 6 μg % in females and a maximum of 260 ± 42 μg % in pregnancy According to Lahey (1953b) the copper level in umbilical cord blood is low 75 ± 14 μg % (average of 14)

Markowitz *et al* (1955) gave the data presented in Table V, (average of ten subjects)

The hypercupremia is due mainly to an increase in ceruloplasmin, but a modest increase in the direct reacting fraction is present as well (Gubler *et al*, 1953, Scheinberg *et al*, 1954, Markowitz *et al*, 1955) An increase in oxidase activity has been demonstrated

Values in infections are not as high as in pregnancy Markowitz *et al*

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Vallee (1952) in a few patients with vascular disease where infarctio questionable or absent were all on the upper side of the mean valu all fell within the range considered normal, which does not contradi above hypothesis Rather large groups of subjects will be necessary t proof of such a minor difference, if it exists The increased globulin le subjects with atherosclerotic disease mentioned above, is also in lin this hypothesis In any case all the patients had an elevated erythr sedimentation rate

The hypocupremia in nephrosis where the albumin globulin ra reversed and total protein levels are low remains to be explained In cases, however, there is a considerable loss by kidney excretion, as has described by Markowitz *et al* (1955) The ceruloplasmin excretion has determined in 4 of his 7 nephrosis patients The loss proved to be as as 16–75 mg ceruloplasmin a day The serum copper values of th patients were between 36 and 129 μg %, with corresponding decrea ceruloplasmin values

Adelstein *et al* (1956) contend that a linear relation exists betwee copper concentration, the ceruloplasmin content and the *p* phenyle amine oxidation in normal subjects (10 subjects), in acute myoc infarction (8 subjects) where a rapid elevation of the levels occurs, a pregnancy (18 cases) with a gradual elevation of the levels How benzidine oxidase levels in myocardial infarction were elevated to a gr extent than could be accounted for by rise of the copper levels alon would certainly be of interest to know these relations in cases of tox of pregnancy The significance of the elevation of the copper and ce plasmin levels and the response of the levels to different circumstanc obscure The elevation in normal pregnancy seems to be due, at least p to the direct or indirect action of estrogens, similar to the other cha in the levels of the blood constituents Estrogen therapy in coro patients gives very high ceruloplasmin levels as well as a normalizin pathological values and ratios of the lipid fractions The two coro victims reported by Russ and Raymunt (1956) reached levels of more t 300 and 500 μg copper per 100 cc on a dose of 1 mg of estrogen a during several weeks, all three xanthomatosis patients reached value 300 μg /100 cc

But this explanation probably cannot hold for the influence of infarc on the copper and ceruloplasmin levels Even if it is supposed that the l is overflowing with toxic products for detoxication and impairment o normal metabolic functions occurs—such as, for instance, the catabol of estrogenic substances—this could hardly be a great enough facto give such long standing elevation of the copper and ceruloplasmin level occurs under these circumstances A more complicated system invol interrelationships with other endocrine glands would be more probable

A passive change of the copper and ceruloplasmin levels together with changes in the globulin levels might be present as well Lipids in the umbilical cord blood show effects similar to those occurring during estrogen therapy, but the ceruloplasmin value does not (Lahey *et al*, 1953a b, Rafstedt, 1955) As has been said, the maternal blood shows high ceruloplasmin levels and no "estrogen" effect on the blood lipids Probably, changes in ceruloplasmin and copper levels can thus be considered as secondary to shifts in the protein levels which may be induced by hormonal action

6 Uric Acid

Uric acid is a compound playing an unexpected role in both diseases Data on the uric acid content of the serum are available for both diseases Harris Jones (1957) described dominant traits for hyperuricemia together with hypercholesterolemia Gertler and Oppenheimer (1953) noted that cardiovascular degenerative disease is far more frequent in gouty patients than in others The serum uric acid levels proved to be statistically higher in males under 40 years of age with coronary heart disease than in a group of similar healthy individuals Adult men (over 18 years of age) have higher levels than women, and a significant positive correlation with the serum cholesterol level exists

In normal pregnancy there is hypouricemia during the first months, with an increase to slightly above the normal near term (Resen Steenstrup, 1956) Mull and Bill (1945) likewise described a hypouricemia in pregnancy Munro Kerr *et al* (1918) noted that the levels of uric acid in toxemia of pregnancy are increased in the absence of severe kidney injury Similar observations were made by Lindeboom (1947) in eclampsia Mukherjee and Govan (1950) reported an elevated level of uric acid in the tissue fluid in the edema of pregnancy in comparison with the tissue fluid of healthy mothers at term

Further according to Adlersberg and Ellenberg (1939) there is some evidence that high fat diets inhibit the uric acid excretion in man (see Harris Jones, 1957)

7 Vitamins Minerals Hormones

Data on the blood levels of vitamins and minerals were discussed in Section II where the effects of the dietary influences were considered The levels of hormones in the blood remain to be considered Though the estrogens are the most relevant few authors have determined the levels of estrogens or related substances in the blood or urine On the other hand, the effects of doses given therapeutically have been described far more extensively, and all available data will be discussed in Section VI

V Deviations from the Normal Amounts of Substances Excreted by the Kidney

Data on this subject are not numerous Urine analyses have been carried out especially in pregnant women, but only rarely in atherosclerotic patients Moreover, as a consequence of the secondary kidney lesion in toxemia of pregnancy, of which the proteinuria is the most outstanding feature, changes in the excretion pattern of other substances are possible

All the data are discussed in other sections Abnormal findings have been reported in connection with a deficient intake of niacin and pyridoxine No changes in choline or methionine excretion could be found, but there were evidences of abnormal findings of another type Further, there is evidence of a lowered excretion of estrogens and of pregnanediol in toxemia of pregnancy The only available urine analyses in normal nonpregnant subjects suitable for comparison are those of Kirk (1951), who reported the amounts of the estrogen and androgen disintegration products in the course of aging, while recently Marmorston *et al* (1958) reported on an estrogen deficit and a decreased 17 ketosteroid excretion in patients who had a myocardial infarction (see next section)

Summarizing the observations on blood and urine analysis, it can be said that as far as the available information for the two diseases can be compared, there is a remarkable similarity There is however, a real need for more knowledge of the "normal" relations and also for simultaneous determinations in patients with atherosclerosis (coronary sclerosis) and patients with a toxemia of pregnancy, living under closely similar circumstances Such knowledge could be acquired by family studies Studies are necessary in population groups where both diseases are rare, as well as in groups where both are frequent, or where one of the diseases is frequent and the other is not While making comparisons, it must also be taken into account that dietary customs may differ between males and females in certain regions, and that different amounts of physical exercise between the sexes can also play a role Extensive studies in women will definitely be required and must concern atherosclerosis as well as toxemia of pregnancy

It must be kept in mind also that in pregnancy, a number of shifts in the blood lipid pattern in the "favorable direction" may occur initially, whereas as toxemia develops, a shift in the reverse direction starts In this way, the disease can be present for some time before the original levels are passed, and the values shift into the "bad direction "

Moreover, uniformity of chemical methods is a first requirement in this respect, and a uniform nomenclature of the lipid and protein fractions is of the utmost importance

VI Some Therapeutic Measures

Therapeutic measures which have been applied in both diseases, other than dietary prescriptions and symptomatic therapy, are few The most important therapy is with estrogenic substances, the next that with thyroid hormone and related substances, and the third that with magnesium sulfate given parenterally The role of hormones in cardiovascular disease has very recently been reviewed by Oliver and Boyd (1958)

1 Estrogens

The great interest in the effects of estrogens in atherosclerosis is due to the observation that women—and especially young women—proved to have some protection against serious complications from atherosclerosis, but after the menopause the disease seems to proceed faster than in men of the same age The effects of estrogenic substances given therapeutically on the blood lipid pattern in men and in coronary victims proved to be very marked A ' normalizing of blood lipid levels takes place the cholesterol level falls, the β lipoprotein level decreases and the α to β lipoprotein and phospholipid cholesterol ratios increase (Barr, 1956, Boyle 1956, Dock, 1956, Robinson, 1956) Barr reported that administration of testosterone exaggerated the plasma abnormalities in subjects with initially normal plasma constituents The effects of testosterone seemed to be augmented by the simultaneous administration of estrogens Changes similar to those on estrogen administration can be seen during the menstrual cycle, but of course the shifts are only slight (Oliver and Boyd 1955a, b) Progesterone does not cause significant changes in the blood lipid pattern (Oliver and Boyd, 1955a, 1956) Castration has effects which can be predicted from the previously mentioned effects of estrogens and androgens (see also Oliver and Boyd, 1958)

Dock (1956) has stressed that massive doses of estrogens cannot give immunity to coronary disease, as is readily seen in men with prostatic cancer treated with estrogens Moreover the side effects of doses effective on the blood pattern are marked if these doses are applied for a considerable time

Apart from the effects on the blood composition estrogenic substances have a definite influence on the vascularity of tissues Estrogen injections can induce uterine bleeding in women in postmenopause Histologically the blood supply to the uterus is increased Holden (1939) demonstrated that one single dose of estrogen in an immature rat causes a hyperemia of the uterine vessels After repeated doses many newly developed arteries can be seen in histological sections (Allen and Masters, 1948) Goldzieher and

Goldzieher (1949) and Kountz (1950) observed the augumented vascularization also in the nasal mucosa and submucosa in women (no data for men are given), and in the skin in men and women Testosterone ointment applied to the skin seemed to have some effect also, but in a much lower percentage of cases than the estrogen preparation Effects on the vascularization of the heart and the great vessels are not reported (and this will hardly be possible), but there is no special reason to suggest that such effects do not occur

Androgen and estrogen excretion data on men and women may indicate whether there is a possible hormonal deficiency According to Kirk (1951) the urinary excretion of neutral 17-ketosteroids—representative for androgen production—declines from about 15 to 4 mg per 24 hours in men between the ages of 30 and 90 years, and from about 10 to 2 mg in women of the same ages This has been confirmed by several authors (Hamburger, 1948, Robinson, 1948, etc , cited by Kirk, 1951) On the other hand the excretion of estrogenic hormones is quite different in the two sexes and is considered to be a reasonably good measure of the estrogen production in the body

Estrogen analysis of the urine reveals practically the same excretion values in young and old men (about 12 rat units per 24 hours), however, the excretion of estrogens is higher in women during the reproductive period of life (35–40 units daily), but drops to only 7 units with the onset of the menopause This has been confirmed by others (Pincus, 1951, Pedersen Bjergaard and Tønnesen, 1948, cited by Kirk, 1951) Marmorston *et al* (1958) reported a substantial estrogen deficit in female patients who had a myocardial infarction, in comparison with healthy and sick controls, judged by the urinary excretion of biologically active estrogens No such effect could be observed in a large group of male subjects (1200), although in a much smaller group investigated previously a deficit could be found The 17-ketosteroid excretion was significantly lower in male and female patients who had recovered from a myocardial infarction than in controls This last finding is not what would be expected But on the other hand, the condition of the patients after an infarction has occurred need not be biochemically the same in all respects as in the condition that caused the infarction

Both in old men and in women in the menopause, the gonadotropin production is considerably increased

There is a relative predominance of estrogens in males with advancing age For instance the androgen estrogen ratio is 13 9 between 17 and 29 years, 7 0 between 50 and 59, 6 4 between 60 and 69, and as low as 2 6 between 80 and 96 years of age (Kirk, 1951) Estrogenic substances are known to stimulate osteoblasts (Kenyon, 1942, with stilbestrol) and to prevent osteoporosis and the deposition of calcium in tissues other than

those normally calcified Aidin (1956) reported a therapeutic effect of stilbestrol on calcified atheroma with regain in elasticity Elkeles (1957) showed radiographically that a correlation exists between calcified atheroma and osteoporosis in men and women over 50, and thus with subsiding hormonal activity

More evidence of a favorable effect on the vessels of slight shifts in hormonal balance can be seen in liver disease where the plasma estrogen level is elevated Although other factors operate here too, liver disease may be a contributory factor in the low incidence of atherosclerosis in some primitive people, for instance the Bantu Moreover there is a higher fibrinolytic activity in the plasma in these cases (Gillman *et al* 1957) Experimentally the fibrinolytic activity of the plasma in rats is modifiable by sex hormones in accordance with the above mentioned observations

A few words are necessary on the influence of hormones on toxemia of pregnancy Estrogen therapy is considered unsuccessful by several authors (see for instance Dieckmann 1948 1952 Dieckmann and Pottinger, 1955, 1956, Dieckmann *et al* 1949, 1951, 1952) However, according to Smith and Smith (1937–1946 Smith 1948) the estrogen levels show a great decrease while there is at the same time a marked rise in the level of the gonadotropins These signs regularly precede the clinical symptoms by 4–6 weeks Urine titrations for these substances were less dependable but a lower sodium pregnanediol excretion was present This excretion pattern was not seen in chronic nephritis or essential hypertension but the toxemia in diabetics behaved like the genuine toxemia Anker (1956) reported a very low estrogen excretion, about 1 mg per liter in a case with severe pre eclampsia, whereas the normal values ranged between 6 6 and 21 4 mg per liter Also the low estrogen levels of the umbilical cord blood are remarkable in toxemia cases The data of Smith and Smith and of Anker were confirmed by Cagnazzo (1956), according to this author the breakdown of estradiolestrone estriol, and further to inactive substances excreted in the urine, is incomplete in toxemia However, other authors could not confirm the findings of Smith and Smith

Smith (1948) and Smith and Smith (1949) could not estimate with certainty the prophylactic value of stilbestrol, owing to the clinical material available There was no control group and only the difference from a "predicted" frequency as described by Mastboom (1948) was considered White (1954), however, reported good results with prophylactic therapy of stilbestrol combined with progesterone in pregnant diabetic women Success seemed to depend on the normalizing of gonadotropin and estrogen levels, and the pregnanediol excretion Vascular disease develops at an early age in these subjects and toxemia of pregnancy occurs very frequently Smith and Smith (1949) suggested that a deficiency of progesterone with a too

rapid and/or faulty destruction of estrogens is present in toxemia According to Russell *et al* (1957) the pregnanediol excretion levels in moderate to severe eclampsia are below normal, in less severe cases the levels are not so low Paine (1957) showed on histological sections of his material that the changes normally occurring in the aging of the placenta are accelerated in cases of toxemia (see also Section VIII)

The endogenous production of estrogens is influenced by a number of vitamins For instance Gyorgi *et al* (1947, 1949) showed that estrogenic substances reduce the need for choline, and also have a distinct lipotropic effect (Griffith, 1940, 1941) A reciprocal influence seems to exist (see Section II, 12 on choline) Nelson *et al* (1951, 1953) could maintain pregnancy in pyridoxine deficient animals if estrone and progesterone were injected As it is known that pyridoxine deficiency can induce toxemia of pregnancy, it is possible that this deficiency interferes with the production of the estrogenic substances

Vitamin E prolongs the estrogenic effect of injected doses of estrogen, but on the other hand vitamin A in high doses has an antiestrogenic effect (Pagliari, 1956, Randazzo, 1956) Moreover a number of vegetable foods, such as wheat, soybean meal, oats, and alfalfa, have a significant estrogenic activity (Pieterse and Andrews, 1956) Thus a vegetable diet might provide some useful dose of estrogenic or related ("pre estrogenic") substances and the same might be true concerning atherosclerosis A lowering of the plasma cholesterol following administration of soysterols has been described (see for instance Peterson *et al*, 1952, 1956) Apart from the competitive effect on the resorption of cholesterol, estrogenic activities might perhaps make some contribution to the effect

2 *Thyroid Hormone*

Thyroid hormone treatment, has sometimes been tried in both diseases, but it does not deserve encouragement because of unwanted and dangerous side effects in which the increased oxygen consumption certainly plays an important role (see for coronary sclerosis, Oliver and Boyd, 1955a, 1956, 1957, 1958, Strisower *et al*, 1957, *Lancet*, 1957, and Symposium on Arteriosclerosis, 1956, and for toxemia of pregnancy, Munro Kerr *et al*, 1948) Many investigations are going on to find related compounds influencing the blood levels favorably but without the dangerous side effects

On the other hand, lack of thyroid hormone due to deficient iodine intake can be a contributory cause for regional high incidences of atherosclerosis such as was demonstrated by Uotila *et al* (1958), Roine *et al* (1958), and Keys *et al* (1958) in Finland Toxemia of pregnancy is certainly not infrequent (Karvonen, 1958a) here, but definite data are not yet at hand

Effects of other hormones reported by Oliver and Boyd (1956, 1958)

include increase of the fibrinolytic activity by the thyroid stimulating hormone, growth hormone, testosterone, and deoxycortisone acetate the effects of which are similar to those of the estrogens Corticotropin and cortisone inhibit fibrinolysis

3 Magnesium Sulfate

Magnesium sulfate is a completely different therapeutic in both diseases It has been used in massive parenteral doses in pre eclampsia for a very long time Good results were reported recently in atherosclerotic patients (see Section II, 15)

It is clear that estrogens alone cannot cure atherosclerosis The same holds true for toxemia of pregnancy As pregnancy has the more direct relation to estrogenic substances, it is also possible that the provision of this factor at a certain time cannot restore all the damage caused by previous lack of it The same argument is true for both diseases, concerning whatever factor has been lacking for some time, it also holds *mutatis mutandis* for a temporary oversupply

It seems probable, however than nonestrogenic substances related to the estrogens may play an important role Something similar can be expected from nonthyroid products related to the thyroid hormone Investigations are being carried out at the moment to find suitable therapeutics of both kinds which do not have unwanted side effects

Since many dietary factors such as vitamins and minerals affect the metabolism of the hormones mentioned here, it can be expected that an absolute or a relative lack of any dietary factor could limit or inhibit normal metabolism The effects can be quantitative as well as qualitative and can yield end products normally absent or nearly so, as well as cause a lack of products necessary for the normal course of metabolic reactions It means too, that under differing circumstances of life, and perhaps more or less regionally, the "factor" which has such a key position" may vary Furthermore, an ample supply of one or more of such dietary factors may influence the necessary amounts of other substances also Sex differences are of course present, and there is a little evidence that the need for some of the vitamins, for instance choline (Griffith, 1940, 1941) and linolenic acid (Greenberg *et al*, 1950 Sinclair, 1956) might be higher for males than for females However, these observations have been made in rats A higher need in pregnant than in nonpregnant women will be obvious

Of the three therapeutics mentioned in this section magnesium sulfate seems to be the most harmless, as it has been used in astonishingly high doses, relative to the plasma level without dangerous side effects It has been used not only in the two diseases under discussion but also in diagnostic procedures such as the measurement of the blood circulation time

This does not imply that increasing the physical activity is a good therapeutic measure once the disease has developed In coronary sclerosis a maximal limit for exercise is set by the symptoms of the disease itself For toxemia of pregnancy the vascular relations are necessarily otherwise From clinical observations in toxemia patients it is known that continuing active life is unfavorable to the patient's health Moreover, N Morris *et al* (1956) demonstrated that the uterine blood flow decreases temporarily on exercise, as by using an "exercycle" in bed An all round physical training should be started before that time "Training"—increasing the physical activity—means a better blood and vessel supply in all the tissues concerned and a much greater general reserve to meet increased demand Moreover, trained muscles perform work with greater efficiency For instance, Karvonen (1958b) reported that the work done by the trained heart at rest was half to two thirds less than that performed by the untrained heart A minimum of exercise is necessary to result in the 'training' effect Estrogens have a definite enhancing action on the growth of new vessels and they may thus be of value in both diseases, apart from their effects on the blood composition (though these effects might be closely connected with each other) Estrogen given during a limited time, together with physical activity—gradually increasing at first and not to be stopped—might be of value as a therapeutic as well especially in coronary sclerosis, but is probably not advisable in clinically established toxemia of pregnancy

8 Stress

Psychical "stress" has often been considered an important cause of the diseases under discussion However, data and results are difficult to evaluate and comparisons of the two diseases are not possible

Some data mentioned by Oliver and Boyd (1956), and Biggs and Macfarlane (1957), reviewing the literature, are of general importance Among the varied influences cited is the fact that experimental stress and adrenaline give an increased fibrinolysis in the same way as exercise, examinations (Latner, 1947), air raids during war, anxiety states, alarming suggestions under hypnosis (Truelove, 1951) etc Further, an increase of the prothrombin concentration (Uvnas 1942, Leibetseder 1951), a decrease of heparin activity (Constantini and Tintori, 1951), and an acceleration of clotting (Donzelot and Kaufman, 1952, Mills *et al*, 1928) have been reported Corticotropin and cortisone inhibit fibrinolysis

An elevating effect on the cholesterol levels and a decreased fibrinolysis and clotting time have been reported by Roseman (1958) in accountants under stress circumstances Byers (1958) inducing stress conditions by electric stimuli in rats, found no change in plasma cholesterol or phos

pholipid levels, but clotting times proved to be shorter On the other hand, the "average" stresses of the war circumstances, however unfavorable these might have been, proved to be more than corrected by other influences such as diet and physical activity

Concluding this section, it can be said that the factor of physical activity among the habits of life certainly is a very important one practically as well as theoretically The factor of psychical stress still remains difficult to evaluate and to investigate

VIII Similarities in Clinical Symptoms

A comparison of these two diseases in respect to the clinical symptoms may seem impossible at first sight However, a few details of the syndromes in both diseases may be mentioned The most evident symptom of toxemia of pregnancy, the elevated blood pressure, is not seen in coronary sclerosis or, at least, not to such an extent [6] However, according to Brown *et al* (1957), the blood pressure in coronary disease, before infarction has occurred, is slightly raised on an average, compared with that in healthy men of the same age After the recovery from the acute phase of an infarction, the blood pressure becomes fixed at a lower level than that in the healthy controls A regulating system to supply the tissues with blood as well as possible is certainly present and substances released from the ischemic and injured tissues must play a role

Considering the consequences of placental ischemia or an infarction, the following course of events could be suggested At first, the blood supply in the placenta and the child's circulation will gradually or abruptly become too low, compensation can come from the mother's circulation [vasoconstrictors, adrenals, or kidney (uterorenal reflex)] Fekete (1958) especially pointed to the fact that hypoxia of the placenta causes a number of products to be formed which act on the blood vessels, resulting in a raised blood pressure which will become still higher with the increasing blood and oxygen need of the growing uterus and its contents When new placental infarctions occur, the demand on the maternal circulation will become still higher There is, obviously, no primary reason whatsoever for a low blood pressure in the mother's circulation when a placental infarction has occurred, as no immediate harm has been done to vital maternal tissue

[6] Hypertension as such does not cause atherosclerosis as has been demonstrated by Kimura (1957) in Japan and by Scrimshaw (1956) (cited by Groen *et al* 1956) in Guatemala In both regions the atherosclerosis incidence is low, whereas hypertension is frequently seen However, if conditions are favorable for the development of atherosclerosis hypertension can promote this development Recently Oliver and Boyd (1958) reviewed the influences that a number of hormones can have on the tonicity of the arteries

as in myocardial infarction Moreover in myocardial infarction there is no need for a blood supply in excess of the normal" After parturition the blood pressure soon becomes normal, since there is no longer any need to force extra blood to the uterus (provided no irreversible harm has been done by a too long duration of the abnormal conditions)

An insufficient blood supply to the placenta is generally accepted as the origin of toxemia in pregnancy and this agrees well with the relatively lower birthweight of the child the tendency to early labor, the small placenta with fewer vessels, etc in the more chronic cases Kloosterman and Huidekoper (1952), found higher placenta weights on an average, in cases of toxemia of pregnancy delivered between the 270th and 290th day of pregnancy It was demonstrated also that the quality of these placentas was poorer as determined by the functional capacity of the tissue per unit weight In heavier placentas there was a greater risk of toxemia and a higher frequency of infarctions In severe cases the placenta weights decreased again The fetal weight decreased progressively with the severity of toxemia This suggests a number of possibilities Growth of the placenta and its size are determined early in pregnancy, certainly earlier than toxemia develops The quality of the uterus mucosa, its vascularity, capacity of 'adaptation" to pregnancy and the vitality of the fetus gemelli etc, will influence the size of the placenta It is a disadvantage for the placenta to be either too small or too large, because of suboptimal reserve capacity (Kloosterman and Huidekoper 1952) Both will then be very sensitive to ischemia—because of too low vascularity either as a result of damage of the vessel walls due to the blood lipid changes mentioned above or by infarctions which are known to occur frequently The ultimate results will be dependent on the initial situation, the time and rate of onset of the pathological changes and the capacity to compensate locally or generally via 'stress" on the mothers' circulation, i e hypertension It will be clear that these effects vary greatly they may result in the death of the child or in premature labor progressing to eclampsia The early and more chronic cases will tend to cause the child or the placenta to be small Subjects with large placentas may do better at first possibly because there are more vessels and narrowing or obstruction does not result in fetal death as early as when the placentas are small

In diabetes, the readily metabolized glucose can be a factor in the known tendency to develop large children and large placentas The tendency to toxemia is then greater for reasons similar to those described above The effects will be greater when the diabetes is poorly regulated resulting in longer periods of raised blood sugar levels each day Distinction has to be made between genuine toxemia cases and those women who develop toxemic symptoms as a result of pre existing vascular disease (Van Bouwdijk

Bastiaanse and Mastboom, 1949, Van Bouwdijk Bastiaanse, 1954) In the latter cases the placenta also does not obtain enough blood and an ischemia will be present, while infarctions may occur as well, but the primary cause of the disease is something else, and therapy should be directed also to that

On the other hand, the above hypothesis does not explain how edema or albuminuria can occur before the blood pressure is raised There may be cases, however, in which the ischemia of the placenta or a possible infarct is not extensive enough to raise the maternal blood pressure immediately Such can be expected when there is an ample supply of vessels initially There may be pathological changes already in the composition of the blood, however The changes in the serum protein pattern, for instance, may cause a greater inclination to develop edema, and the hormonal conditions in pregnancy also increase this tendency

As to edema, an inadequate supply of choline can be important (Bloem and Neumann, 1953) In one of the cases described (a nephrosis patient), the albuminuria, which was high initially, became "negligible" after the edema had disappeared on choline therapy Moreover, in pregnancy, in some cases, there may be an orthostatic albuminuria as well

It will be clear that in cardiac infarction it is impossible to get a higher blood pressure than before, even if this should be required for the normal blood supply of the tissues In contradistinction to pregnancy, the amount of blood or oxygen that has to be supplied does not increase There may be only the effect of the greater force necessary to propel the blood through the vessels narrowed by atherosclerotic processes, but this effect develops very slowly and is independent of the infarction Vascular spasms, on the contrary, may greatly increase the force necessary for blood supply of the tissues and thus increase the blood pressure

In pregnancy with placental ischemia or infarction, the "pump" has not been damaged and the maternal vascular system can maintain for a time a higher "activity" This state cannot continue indefinitely without doing harm to the maternal tissues, for instance the kidneys Spasms of the uterine or other vessels may also occur (see for instance Fitzgerald and Clift, 1958 Fekete, 1958) The later symptoms—those of the real preeclampsia and eclampsia—should be entirely secondary then, and they are certainly very similar to the symptoms and course of events in diseases of another origin where a rapidly rising blood pressure, with possibly an overfilled circulation, also occurs, as in malignant hypertension Salt restriction obviously inhibits these secondary symptoms, but, as is well known, is not curative That the raised blood pressure really benefits the child can be seen by the unfavorable effects which hypotensive drugs, given to the mother, may have on the unborn child

Walker and Turnbull (1953) and Mackay (1957), by measuring the oxygen levels in the cord blood, have demonstrated that oxygen supply is reduced in toxemia cases MacKay also reports an increase in the occurrence of fibrin nodes, which has often been commented on by other authors as well although quantitative measurements are scarce Moore and Myerscough (1957) and also Morris *et al* (1955) found a reduced clearance rate and reduced blood flow of the myometrium in pre eclampsia, while Browne and Veall (1953) demonstrated a reduced clearance rate in the chorio decidual space It is widely believed that this lowered blood flow and thus lowered oxygen supply to the placenta is associated with a degeneration of the placental membrane Evidence for this is the higher hemoglobin and erythrocyte count in the fetal blood in cases of maternal toxemia of pregnancy Moreover Van Bouwdijk Bastiaanse and Mastboom (1949) were able to produce an experimental toxemia of pregnancy in animals by partially ligating the uterine vessels in such a way as to produce a uterine and placental ischemia

Fekete (1958) points to the fact that hypoxia of the placenta causes the formation of a number of substances which can act on the blood vessels, these include histamine tyramine, acetylcholine, serotonin guanidine, arginine thromboplastin, and others This may explain vascular spasms, higher thrombotic activity and pre eclampsia eclampsia, as well as the occurrence of a striking rise in blood pressure in the first few days of the puerperium whereas blood pressure was normal antepartum This latter effect has also been observed by the author in an Amsterdam clinic The effect will last as long as the substances acting on the blood pressure persist, which may be from some minutes up to a few days (Fekete, 1958) and is also dependent on the presence of counteracting substances

Paine (1957) in histological preparations of placentas of toxemia patients, observed a premature syncitial atrophy accompanied by a reduction in syncitial lipid which relates to the generally lowered values for pregnanediol excretion The abnormal changes are not seen in cases where acute toxemia manifests itself only in the last few weeks of pregnancy In accordance with this vascular changes are more marked where there is pre existent hypertension so that the vascular system has to suffer even early in pregnancy Thromboses in the decidual veins and hemorrhages were also common Slow depositions of fibrin from the maternal circulation could be seen, and these might result in occlusion and infarct formation although a residual fetal circulation through the villous cores was present This picture seems to be not unlike the course of events in coronary atheroma formation as given by Levene (1956) Duguid (1956) and Duguid and Robertson (1957)

Infarctions are very frequently seen in the placentas of toxemia cases

whereas they are less frequent in clinically uncomplicated pregnancies Young was the first to report this fact, as far back as 1914 (see also Young, 1927, Morris *et al*, 1955) Zeek and Assali (1950) report a strong correlation, in 83% of the 59 toxemia cases, true placental infarcts were present, whereas they were found only in 16% of the 122 nontoxemia cases Bartholomew *et al* (1957) report very similar incidences The variations in the incidences reported, and the more or less distinct relation of infarctions to toxemia of pregnancy (see also Dieckmann, 1952), may well be due to cases in which no ischemia developed severe enough to give rise to clinical symptoms It may also be that a severe enough ischemia could develop without an infarction having occurred, either because the supply of blood vessels was too limited, or because of pre existent atheromatous changes of the uterine vessels Fatty subendothelial deposits, suggesting atheromatous changes, could also be demonstrated This was confirmed once more by Bartholomew *et al* (1957), who also pointed to the important role of vascular spasm with subsequent infarction According to Bartholomew *et al* spasms are the most probable cause of the ischemia and subsequent thrombosis, though thrombosis as an initial event could not be ruled out However the spasms these workers observed were in the placental veins, in such a way *that the smooth muscle sphincters (discovered by Spanner) obstruct* the exit of fetal blood from the corresponding placental unit Bartholomew *et al* believe the spasm is caused by oxytocin, as a result of hormonal imbalance The obstruction causes distension of the villous capillaries, enlargement of the villi, diminution or obliteration of the intervillous spaces and decreasing circulation By the compression of the tissues, anoxemia and necrosis result Thromboplastin, the first product released from the necrotic tissues, causes clot formation Once a clot has been formed, other toxic substances from autolytic processes begin to act Moreover, ischemic tissues also release toxic substances to the circulation As the fetal blood does not show the biochemical changes, resulting in enhancement of the tendency to clotting, *as occur in the blood of the mother, the spasms in the fetal veins* described by Bartholomew *et al* are indeed likely to occur before the thrombosis However, vasoconstrictors released from ischemic tissues on the maternal side might perhaps contribute to the occurrence of these spasms Similarly Schneider and Engstrom (1954) and Schneider (1955) point to the release of thromboplastin on autoextraction of blood clots The effect this has on the inclination to coagulation, when it has been released from a retroplacental hematoma, is even sufficient to cause fibrin deposits in the pulmonary circulation which can then give the symptoms of an acute cor pulmonale, and can result moreover in defibrination of the blood and subsequent tendency to hemorrhage Furthermore, according to *Schneider and Engstrom*, 1954, and Schneider, 1955, the serotonin released

from the blood platelets is a strong vasoconstrictor and immediately increases the blood pressure In addition to serotonin a number of other active substances come into the circulation It should be remarked however, that according to Page (1954) serotonin injected intravenously into human beings can give a variety of effects such as hyperpnea, tachycardia, itching, burning sensations, etc , but its effect on the blood pressure is not consistent Correspondingly, patients with argentaffinoma producing large amounts of serotonin do not always have a high blood pressure Very probably, thus, the induced reactions and responses are extremely complicated

Something more should be said about the retroplacental hematoma and abruptio placentae This complication of pregnancy is accepted as equivalent to toxemia of pregnancy in its more usual form by many authors (Holmer *et al*, 1956, Bartholomew *et al*, 1957, Russell and Dewhurst 1958), although others divide these cases into a nontoxic and a toxic group the former showing no signs of toxemia before the abruptio placentae, while the latter do In view of the suggested etiology of toxemia signs of a toxemia may sometimes appear before an abruptio placentae occurs It can be expected that there are women without clinical signs who already have the changes in the blood composition, described in the previous sections which increase the tendency to blood clotting and atheromatous changes in the intima of the vessels and can result finally in obstruction of these vessels If then a placental infarction occurs, extensive enough, or extending rapidly enough, with secondary hemorrhage a seemingly nontoxic case could be found Of course this cannot exclude the possibility of real nontoxic cases in which the cause is something else

It only remains to be explained why the infarction occurs then in the placental arteries and not in the coronary vessels or elsewhere No definite answer is possible at the moment Certainly the caliber of the vessels plays a role in the amount of deposits necessary to result in a distinct diminution or obstruction of the lumen In addition to the vascularity, the rate at which oxygen is absorbed from the blood is important, especially when a large enough supply of blood to keep the oxygen at an adequate level is not possible A low oxygen content of the blood and thus of the vessel wall will certainly limit the capacity to eliminate and withhold "unwanted" and harmful blood constituents That the harm done to the vessels in pregnancy does not involve the placental vessels exclusively is suggested by the findings of Oliver and Boyd (1955a), who noted that a group of postmenopausal patients with coronary disease had had more pregnancies than a comparable control group who had no coronary disease

The pathological changes in the placental vessels can be very distinct Zeek and Assali (1950) even saw such a striking resemblance to atheroma

tosis in the coronary vessels or aorta that they described their findings in the decidual vessels, present in a high proportion of toxemic cases, as an "acute atherosis" The same was true in the vessels of the parietalis The next most frequent finding was retroplacental hemorrhage In addition, secondary hemorrhages in the infarcts were often seen, they frequently extended beyond the infarct into the retroplacental decidua, causing premature separation of the placenta The atherosis lesion was characterized by the deposition of large amounts of fatty material in the intima of the decidual vessels Obstruction of the lumen occurred later on The more advanced stages were accompanied by fibrinoid necrosis and a pleomorphic type of inflammatory exudate which caused further obstruction of the lumen This form of acute atherosis was found in endometrial spiral arterioles, in their decidual termini, and in endometrial venous lakes The latter are considered important for the regulation of the endometrial blood flow, they have both arterial and venous connections The acute atherosis lesion is a diffuse lesion of the decidua and seemed to be "most marked in the decidua basalis near areas of ischemic necrosis of decidua and overlying placental infarcts" Regressive changes could be found in five of the toxemia patients who succumbed at various intervals after delivery Regression seemed to start immediately after delivery Within 5 weeks "the lesions were indistinguishable from those of ordinary atherosclerosis except for their limited distribution and the unusual degree of severity in young individuals"

Of the whole series of 232 cases, including 9 cases of pregnant or puerperal uteri removed surgically, and tissues from 30 autopsies, acute atherosis was found in 34 of the 71 toxemia patients and in only 3 of the 143 non toxemic nonhypertensive patients In these latter 3, the lesions were early and not yet obstructive Furthermore "acute atherosis" was not seen in any of the non-toxemic hypertensive patients In this group, however, moderate hyperplastic arteriolar sclerosis, a lesion commonly associated with essential hypertension" was frequently observed

Recently Robertson and Dixon (1958) also reported intimal changes with narrowing of the lumen of the maternal placental blood vessels in preeclampsia Obstructions in the circulation of the fetal part of the placenta were noted also by Zeek and Assali (1950), though there was no correlation with toxemia of pregnancy The lesions were characterized by acute angiitis and proliferative endarteritis A similar acute angiitis was also present in the maternal vessels in some cases The endarteritis was "evidenced by an increase in the various cellular components of the intima resulting in a thickened, loose meshed, cellular tissue which often presented a foamy appearance and which caused narrowing of the lumens" This lesion was considered as possibly identical with the lesions described as occurring

most frequently in cases of toxemia of pregnancy by Bartholomew and Kracke (1936) and by Hunt *et al* (1940), who characterized them as cholesterol arteriosclerosis Zeek and Assali (1950) could, however, not demonstrate lipid material in the lesions they observed The lesions were most marked in cases in which death of the fetus had occurred *in utero* long before delivery

Bartholomew and Kracke (1936) reported that the atheromatous changes observed were mainly in the arteries of the medium sized villous stems less frequently in the large arteries of the main stems, and very seldom in the vessels or capillaries of the terminal villi Thrombosis and infarction occurred and a great similarity was seen to the development of coronary thrombosis in man as well as to the experimental coronary lesions induced in rabbits Hunt *et al* (1940) reported furthermore that pregnant rabbits, thyroidectomized at the end of the first trimester, died in convulsions near term The placentas of these animals appeared to have infarctions and a severe cholesterol endarteritis These rabbits did not develop hypercholesterolemia, as nonpregnant rabbits do but their fetuses did

An observation possibly related to these problems has been made by Popják (1954), studying the placental transfer of lipids in experiments with rabbits Pregnant animals fed a cholesterol diet showed the hyperlipemia that usually occurs in this species on such a diet, but nothing has been said on the development of possible toxemia Plasma phospholipid levels in fetuses from cholesterol fed mothers were slightly higher than in the controls Nevertheless, as Popjak says, this does not mean a transfer of excess of lipid from the mother to fetus, "because the cholesterol feeding produced an abnormal condition in the placenta in that its fetal part was impregnated with cholesterol and its esters and, moreover, this change interfered seriously with fetal development resulting in fetuses one third lighter than normal" It is, however, very difficult to make comparisons between man and the rabbit since it is known that their lipid metabolism is somewhat different

The course of events in myocardial ischemia and infarction is simpler to describe than that of placental infarction There are similar lipid deposits in the vessel walls, there is ischemia by which vasopressor agents can be released in the circulation, spasms can occur, and the biochemical relations within the blood complete the picture with a higher tendency to clotting An infarction may result more vasopressive substances come into the circulation, etc The extension of the infarct and a possible secondary hemorrhage, just as in a placental infarction determine the final outcome ranging from cases missed clinically or slight cases to acutely fatal ones

There are a few additional points Tampan *et al* (1956) suggested a hypertrophy of the adrenal cortex induced by vitamin B deficiency as the

chemical as well as histological, show a striking similarity It can be objected that a number of these pathological signs may be completely nonspecific and that they might occur as well in a number of other diseases caused by a deficiency of one or more of the factors under discussion Furthermore, individual susceptibility, i e heredity, and different imbalances in the deficiencies will influence the end result the clinical signs

Known relations with other diseases (a number of them sometimes called "civilization diseases") are present, for instance, via the essential fatty acids, a deficiency of which has been described also in idiopathic hypercalcemia in infants (Sinclair, 1956, 1957, 1958a, Frazer 1956, Bongiovanni *et al* 1957) These authors also reported in this connection a greater susceptibility to vitamin D intoxication A deficiency of essential fatty acids, and good responses from their administration, have been noted by Hansen (1933, 1957) and Hansen *et al* (1947) in infant eczema Treatment of exudative diathesis in children of a somewhat older age by avoiding animal fats is often successful, if properly done That the functions and effects of the (essential) unsaturated fatty acids are not completely the same is demonstrated clearly by the fact that linolenate cannot cure the dermal symptoms in rats on a fat deficient diet, whereas linoleate does so readily Other symptoms can be relieved by both

Diabetic animals develop signs of essential fatty acid deficiency earlier than normal ones on a diet deficient in essential fatty acids (Peifer and Holman, 1955) In the diabetic a reverse relation is present between the *essential fatty acid content of the blood and the glucose level Furthermore,* it is well known that diabetics develop atherosclerosis easily, while diabetic women are known to suffer frequently from toxemia of pregnancy also The prevalence of both diseases in diabetics might be related to the endocrine disorder itself but could equally well be due to the dietary measures Especially the low carbohydrate high fat diet formerly so commonly described, must be considered as harmful in this respect Even the resistance to radiation sickness is less in essential fatty acid deficiency states (Decker *et al*, 1950, Cheng *et al*, 1954, 1955, 1956, Deuel *et al*, 1953)

Recently Sinclair (1958b) pointed to the influence of the essential fatty acids on arcus senilis In rats, arcus senilis could be produced by an essential fatty acid deficiency, and improvement occurred when these fatty acids were again supplied Apart from the relations between lipid deposits in arcus senilis and atherosclerosis, there is some evidence of a regional distribution similar to that of toxemia of pregnancy Duncan (1947) reported a low incidence of toxemia in the Subarctic at Dawson, Yukon, while atherosclerosis was reported to be infrequent also among the people of advanced age Sinclair (1958b) found the incidence of arcus senilis in Canadian Eskimos and Canadian Indians extremely low, even in advanced age, whereas

as among the young men carefully selected for pilot training from Canada and New Zealand (about 22 years of age) 10% had arcus senilis In the regions these men came from, toxemia incidence is certainly not low Welin and Cramèr (1957) observed that arcus senilis is a symptom indicating poor diagnosis in a group of Swedish patients with hypercholesterolemia and angina pectoris

Dental caries are said to be influenced favorably by vanadium (Rygh, 1949, 1951, Geyer, 1953, Munch, 1957) and Winiker (1957) states an optimal dose of vanadium exists Moreover, it has been stated by Curran (1954) that vanadium decreases the synthesis of cholesterol by the liver and the synthesis of fatty acids from acetate Iron as ferrous salt has the same effect, but in somewhat lesser degree (N B in anemia a hyperlipemia is present) but manganese and chromium have the opposite effect, increasing the cholesterol synthesis in the liver Curran and Costello (1956) were also able to demonstrate that when vanadyl sulfate was given to rabbits, fed prior to this period with cholesterol, it reduced the excess of cholesterol deposited in the aorta Bernheim and Bernheim (1939a,b) found that vanadium markedly increases the oxidation of phospholipids in the liver, in which process a desaturation of the fatty acids of these phospholipids occurs with a demonstrable increase in the amount of double bonds Manganese inhibits this effect too *β* Lecithins are more easily attacked than *α* lecithins while the cephalins in soybean phospholipids are more rapidly attacked by the liver than is the lecithin fraction this might be important in respect to the action of the cephalin in promoting blood clotting Both authors tested other metals, but these had no activity in the reactions studied by them

Experimental pyridoxine deficiency causes dental caries as well as atherosclerosis Dental caries increase as a result of civilization and availability of refined and preserved food in general (see Scott, 1956) Wartime circumstances reduced dental caries (Toverud, 1956 1957) as well as death from coronary sclerotic diseases and the incidence of toxemia of pregnancy Cirrhosis of the liver has been mentioned also as a sequel of pyridoxine deficiency The role of a deficiency of lipotropic factors such as choline methionine inositol, etc , in this respect has been known for a much longer time Feeding of vitamin B_{12} can make the symptoms of atherosclerosis much worse (Annand, 1957) Pantothenic acid deficiency results in a lowering of the cholesterol level in man (Bean *et al* , 1955)

Osteoporosis is connected especially with a diminished estrogen production in advanced age Estrogens stimulate the osteoblasts and prevent calcium deposits in other tissues They also stimulate nitrogen retention and increase protein synthesis in the bone matrix Testosterone also has the latter activities

Tissue necrosis may bring about shifts in the blood lipid levels similar to

those reported for atherosclerosis and toxemia of pregnancy Elevation of the levels of a number of enzymes has been seen in cardiac infarction as well as in necrosis of other tissues

In connection with the usual therapy in coronary thrombosis and the reported evidence of an elevated uric acid level in coronary disease, it might be remarked that *dicumarol increases the urinary excretion of uric acid* with subsequent fall in the serum uric acid level (Hansen and Holten, 1958)

If the relations between toxemia of pregnancy and atherosclerosis are really as close as is suggested by the above findings, a more extensive study of the biochemistry, anatomical pathology, and incidence of toxemia should provide valuable information for atherosclerosis Toxemia of pregnancy is a disease of limited duration and provides a very valuable pathologic anatomical substrate in the placenta Furthermore, studies of the incidence of atherosclerotic disease in these subjects, at a more advanced age, in comparison with that in normal controls, should prove very informative Oliver and Boyd (1955a) have made one such study

In conclusion, it may be said that in both diseases a hypoxia develops as a result of faulty metabolism A primary cause of this seems to be a borderline deficiency of a great number of factors, together with a relative excess of a few others *Quantitative data cannot yet be given The results* are a disturbance of the metabolic processes, whether directly by the deficiency or the excess and/or by a subsequent deposition of substances that cannot be broken down, and/or by indirect damage

It is not surprising that so many factors are involved since all of them influence others or modify the activity of the substances produced In the whole chain of metabolic reactions leading to the necessary end products, such as heparin or the sex hormones, and closely related substances, many factors are involved Any vitamin (acting as an integral part of one of the enzymes involved), any mineral (acting as catalyzer, or being a constituent of the tissues), or any other "building stone" which is present in such small amounts that one or more metabolic processes are inhibited, disrupts the whole chain of events, so that the relative lack of any one of a large number of substances can give the same or a similar end result The "weakest" (most *deficient) link in the chain of reactions limits* the "*strength*" or its yield

The only method for the prevention of these diseases should be, then, the supply of those factors which are generally lacking *and the reduction* of the consumption of those generally supplied in excess Supplying only one or two of the deficient factors will be effective only when these were the "limiting" factors in the rate of the metabolic process(es) involved *for* the subject concerned Of course there will be individual variations in the

need of the various factors, and the habits of life, such as the amount of physical activity, will influence the demand for certain end products

This review does not pretend to solve the problems under discussion It is intended rather to point to the striking similarities in the possible causes and pathology of atherosclerosis and toxemia of pregnancy A simultaneous study on both these diseases together, and including a number of related conditions, would be highly desirable To obtain information on all the factors involved, qualitatively as well as quantitatively, extensive studies concerning the incidence of the diseases in different areas, the true normal relations the biochemical and anatomical pathology the influence of dietary and other measures, are of the utmost importance

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Progestational Agents and the Control of Fertility

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I Introduction

In mammals the physiology of reproduction may be considered as having two aspects (1) mechanisms concerned with the production of the germ cells, and (2) processes for the safeguarding of the mature germ cells and the conceptus The proper conclusion of each of these aspects is brought about by specific hormonal regulation Thus the full maturation of the spermatozoa depends upon circulating pituitary gonadotropin and probably also upon steroidal compounds produced within the testes as the result of gonadotropin stimulation Although the ova may develop to the brink of maturation independent of pituitary hormone secretion, the growth and rupture of the follicles in which they reside is gonadotropin dependent Gonadotropin is essential for adequate steroid hormone production by ovaries and testes, and the steroid sex hormones in turn are regulators of the growth and activity of the accessory organs of the reproductive tract In the female ovarian hormones determine the orderly progress of the fertilized ova through the oviducts, the implantation of the conceptus, the maintenance of the fetus and its expulsion at parturition Because of their effects on oviduct contractions, the ovarian hormones affect the nature of sperm transport in the female reproductive tract, and even the maturation of sperm into fertilizing agents appears to be in part conditioned by sex hormone action upon the uterus (see below)

The maintenance of normal fertility in mammals is thus a complex of processes subject at almost every step to a balanced sequence of hormonal regulation Infertility may arise because of a fault in any single step in this complex, but its etiology may be complicated either by the multiple effects

of a single defect (e g hyperestrinism in the female may affect ovum and sperm transport, the nature of uterine endometrial development, the state of the cervical mucus, the vaginal milieu) or by the diverse effects of alterations in the pituitary-gonad relationship The gonad hormones are regulators not only of the organs and tissues of the reproductive tract but they also affect the secretion of their regulatory pituitary hormones It is this effect of ovarian hormones upon pituitary secretion that is primarily responsible for cyclicity in the female and for the inhibition of such cyclicity during gestation

The foregoing considerations are commonplaces, these, however, are essential to studies of natural or induced infertility And if one seeks to induce infertility by hormonal agents, the repercussions as well as the immediate effects of such action must be realized The simulation of a normal physiological mechanism associated with temporary sterility would therefore be a desideratum for such induced infertility, and it is for this reason that we turned to a consideration of hormonal conditions in the luteal phase of the sex cycle and in early pregnancy Here the ascendancy of progesterone secretion is associated with a lack of ovulation That such ovulation inhibition may be effected by exogenous progesterone had been demonstrated for rabbits in 1937 by Makepeace *et al* and for rats in 1939 by Astwood and Fevold Presumably this ovulation inhibition by progesterone accounts for the absence of superfetation in pregnancy, and a suppression of the secretion of ovulation inducing amounts of pituitary gonadotropin occurs In rabbits prevented from ovulation by progesterone administration, Pincus and Chang (1953) found that the administration of pituitary gonadotropin led to ovulation with a 10 hour latent period following gonadotropin injection, which is exactly that observed following endogenous gonadotropin release The induction of a pseudopregnant state by progesterone administered before expected ovulation seemed therefore to offer a simple means of inducing temporary sterility That such a sterility would be temporary seemed to be assured by the return to normal ovulating capacity in progesterone treated rabbits (Pincus and Chang, 1953) and indeed in the ovulation seen following normal pseudopregnancy

II Progesterone in Human Subjects

The induction of an ovulation free pseudopregnancy in women was attempted in a group of normally ovulating, cyclic subjects with the use of progesterone taken orally (Pincus, 1956) Oral progesterone was used to avoid the necessity of repeated injections The regime of administration involved the daily taking of 300 mg of progesterone beginning on the fifth day of the menstrual cycle and continuing until the twenty fifth day This regime was used to attain two objectives (1) to have progesterone medica

tion instituted before ovulation (ovulation in women ordinarily occurs at mid cycle, but may occur quite early in some cycles), and (2) to sustain the uterine endometrium during medication so that a menstrual flow would occur following cessation of medication In order to assess the effects of the medication, three types of observation diagnostic of ovulation were made on each subject wherever possible (1) daily recording of basal body temperatures, (2) daily vaginal smears, and (3) an endometrial biopsy taken during the time of the normal luteal phase ordinarily on the twen tieth to twenty third day of the cycle Each subject was observed during a control menstrual cycle which preceded the medication cycles Treat ment cycles varied from one to four, but in most cases were three The observations made on these subjects are summarized in Table I They indicate an average shortening of menstrual cycle length (by 2 4 days)

TABLE I

EFFECTS OF ORAL PROGESTERONE ON THREE INDEXES OF OVULATION

Medication	Number of cycles	Mean cycle length (days)	Per cent positive for ovulation by		
			Basal temper ature	Endo metrial biopsy	Vaginal smear
None (control)	33	27 9 ± 0 61	100	100	100
Progesterone	69	25 5 ± 0 59	27	18	6

during medication, and a marked reduction in the frequency of ovulation as judged by the three indexes Since progesterone is thermogenic and has characteristic effects on the uterine endometrium and vaginal epithelium, the alterations in these indexes may represent a direct action of proges terone and may not be reflective of the hormonal consequences of ovula tion inhibition and the ensuing lack of corpus luteum formation

On the basis of an analysis of these indexes taken together, we concluded that ovulation inhibition occurred in 85% of the first cycles of treatment and in 95–100% of the second and third cycles, as though some delay occurred in the establishment of a complete antiovulatory effect (Pincus, 1956) Laparotomies performed at the appropriate time on women during a first treatment cycle disclosed that 70% had failed to ovulate Ishikawa *et al* (1957) employing the same regime of progesterone administration also observed suppression of ovulation in a proportion of the cases taken to laparotomy Although sexual intercourse was practised freely by the sub jects of our experiments and those of Ishikawa *et al*, no pregnancies oc curred Since ovulation presumably took place in a proportion of cycles,

the lack of any pregnancies may be due to chance, but Ishikawa *et al* (1957) have presented data indicating that in women receiving oral progesterone the cervical mucus becomes impenetrable to sperm

The disadvantages of oral progesterone as an antifertility agent are (1) the high dosage necessary to ensure a clear cut effect, and (2) the rather high frequency of occurrence of premature menstruation—in 18% of the medication cycles listed in Table I there was a breakthrough bleeding In a subsequent study we have attempted to reduce the frequency of breakthrough bleedings by supplementing oral progesterone with an oral estrogen (Pincus, 1957, 1959) Estrogen supplementation appears to inhibit breakthrough for a few cycles, but eventually this is overcome and a succession of short cycles ensues, averaging after nine cycles of medication 17 6 days, compared to a pretreatment length of 27 9 days A need for more potent orally effective compounds with improved cyclic regulatory activity was indicated

III Newer Progestational Agents

In studying the effects in the rabbit of a large variety of steroid compounds, we were struck by the finding that four of them which were quite active by both oral and subcutaneous routes had quite similar structual configurations Each of these compounds was classed as a 19 nor steroid since it lacked the angular methyl group containing carbon-19 They were 17α ethynyl 19 nortestosterone (*I*), 17α ethynyl estra 5,10 eneolone (*II*), 17α ethyl 19 nortestosterone (*III*), and 17α methyl 19 nortestosterone (*IV*) With oral administration, compound *I* had been reported by Hertz *et al* (1954) to be five times as active as 17α ethynyltestosterone in the conventional Clauberg assay of progestational activity in the rabbit, and later was found to be about four times as active in women (Hertz *et al*, 1956) In 1956 we reported on the comparative biological activity of progesterone and compounds *I* to *IV* in six types of test (progestational potency by Clauberg assay, ovulation inhibiting action and implantation sustainment in rabbits, deciduomagenic and antifertility potency in rats, and uterotropic action in mice), and found that only compound *III* tended consistently to parallel the action of progesterone (Pincus *et al*, 1956a, b) Compound *II* proved to be weakly estrogenic, and *I* and *II* were relatively ineffective in sustaining implantation in the rabbit and deciduoma *in the* rat We also reported on the effects of orally administered *I*, *II*, and *III* on the normal menstrual cycle in women (Rock *et al*, 1956) Greenblatt (1956) found compound *I* to be twice as active as ethynyltestosterone as an inducer of withdrawal bleeding in women In the same year, Overbeek and de Visser (1956) reported on the biological activities of *IV*, and Ferin (1956) found it to be fifty times as active as ethynyltestosterone as an

oral progestin in ovariectomized women Also in 1956 Heller found *I* and *II* to be singularly potent suppressors of premenstrual tension in women

Since 1956 the number of steroid compounds found to have significant oral progestational activity has increased geometrically A list is presented in Table II of those which have been studied in some detail The conventional test for the progestational potency of these compounds is the response of the endometrium of the immature, estrogen primed rabbit (Clauberg assay) Since this test involves a subjective judgment of the degree of pseudopregnant proliferation seen in an appropriately stained microscope section (Clauberg, 1930), we have sought a more objective method for the quantitative evaluation of progestational activity and have compared dosage response curves derived by planimetric measurement of the degree of glandular development in uterine sections (Pincus and Werthessen, 1937) and by measurement of uterine carbonic anhydrase concentrations in Clauberg rabbits (Pincus *et al*, 1957) This effect of progesterone on endometrial carbonic anhydrase, originally described by Lutwak Mann (1955), proved to be as accurate as the microscopic measurement, easily quantifiable, and amenable to conventional dosage response analysis Accordingly, we have used this method to determine the relative potency of a group of the compounds listed in Table II (Miyake and Pincus, 1958) Taking the oral potency of compound *VIII* as 1 0, we find the relative oral potencies listed in Table III

If progestational potency as judged by this assay is an index of fertility controlling potency, we would seem to have a formidable array of orally active compounds The especially high potency of two of them (*IX* and *XII*) would seem to assure us of especially effective substances Furthermore, in the rabbit the oral effectiveness of these compounds as ovulation inhibitors roughly parallels the progestational efficiency (Pincus and Merrill, 1959)

IV Newer Progestational Agents in the Normal Menstrual Cycle

Using the regime of administration described for oral progesterone, we have studied the effects of compounds *I*, *II*, and *III* upon the menstrual cycles of regularly ovulating, normally cyclic women (Pincus, 1958, 1959 Rock and Garcia, 1957, Rock *et al*, 1956, 1957) Since daily dosages of 10 mg and above gave entirely similar results, we present in Table IV the mean data for cycle lengths, indexes of ovulation, and steroid excretion obtained with subjects studied in 50 premedication (control) cycles and 112 medication cycles Again on the basis of the ovulation indexes, a reduction of 88%–100% in ovulation occurred during cycles of lengths which did not differ significantly from those in the controls In contrast to an insignificant decline in 17 ketosteroid output, a very marked reduction in

TABLE II
Oral Progestins

Number	Compound	References
I	17α Ethynyl 19 nortestosterone	1, 7, 9, 10 14, 15, 19, 20, 22, 23, 27, 30
II	17α Ethynyl estra 5(10)ene 17β ol 3 one	10, 16, 18 20, 22 23 27 28
III	17α Ethyl 19 nortestosterone	7, 10, 20 22, 23 27, 29
IV	17α Methyl 19 nortestosterone	6, 7, 17 20, 21, 22 23
V	17α Propyl 19 nortestosterone	20, 26
VI	17α (1 Methallyl) 19 nortestosterone	20, 27
VII	17α Allyl 19 nortestosterone	20
VIII	17α Hydroxyprogesterone 17 acetate	20, 29
IX	17α Hydroxy 6α methylprogesterone 17 acetate	11, 20, 24 29
X	9α Bromo 11 ketoprogesterone	8, 10 29, 31
XI	6α 21 Dimethylethisterone	2, 12
XII	17α (2 Methallyl) 19 nortestosterone	5 25
XIII	9α Fluoro 11β hydroxyprogesterone	8
XIV	12α Bromo 11β hydroxyprogesterone	8
XV	19 Nor 3 (3 oxo 17β OH-4 androsten 17α yl) propionic γ lactone	13
XVI	17α Vinyl 19 nortestosterone	3 26
XVII	17α Ethynyl 21 methyltestosterone	3 12
XVIII	17α Ethynyl 21 ethyltestosterone	3, 12
XIX	17α Methylestra 5(10) eneolone	3 28
XX	17α Ethylestra 5(10) eneolone	3 28
XXI	17α Propylestra 5(10) eneolone	3 28
XXII	9α Bromo 11β hydroxyprogesterone	8
XXIII	9α Chloro 11β hydroxyprogesterone	8
XXIV	9α Fluoro 11 ketoprogesterone	8
XXV	9α Chloro 11 ketoprogesterone	8
XXVI	12α Chloro 11β hydroxyprogesterone	8
XXVII	21 Fluoro 11α acetoxyprogesterone	3 4
XXVIII	6α Methyl 17α ethynyltestosterone	3 12
XXIX	6α Methyl 21 ethyl 17α ethynyltestosterone	3, 12

Key to references

1 Baquero (1957)
2 David *et al* (1957)
3 Drill (1959)
4 Elton (In preparation) (1959)
5 Elton and Edgren (1958)
6 Ferin (1956)
7 Ferin (1957)
8 Fried *et al* (1958)
9 Greenblatt (1956)
10 Greenblatt (1958)
11 Greenblatt and Barfield (1959)
12 Hartley *et al* (1957)
13 Hertz and Tullner (1958)
14 Hertz *et al* (1954)
15 Hertz *et al* (1956)
16 Kistner (1958)
17 Kotsalo (1957)
18 Kupperman and Epstein (1957)
19 McGinty and Djerassi (19 8)
20 Miyake and Pincus (1958)
21 Overbeek and de Visser (1956)
22 Pincus (1956a)
23 Pincus *et al* (1956b)
24 Sala (1958)
25 Saunders (1958)
26 Saunders *et al* (1957a)
27 Saunders and Drill (1958)
28 Saunders *et al* (1957b)
29 Stucki (1958)
30 Tullner and Hertz (1957)
31 Wied and Davis (1957)

pregnanediol excretion took place, indicating the reduction of progesterone secretion to preovulatory levels (Pincus *et al*, 1958a) Actually, in a series of laparotomies conducted with patients receiving compound *I*, no newly

TABLE III
PROGESTATIONAL ACTIVITY BY ENDOMETRIAL CARBONIC ANHYDRASE ASSAY OF A GROUP OF PROGESTINS

Compound number	Relative potency by oral administration
I	1 0
II	0 3
III	3 2
IV	1 5
V	1 7
VI	1 3
VII	1 3
VIII	1 0
IX	13 6
XII	14 5[b]

See Table II
[b] Unpublished data

TABLE IV
EFFECTS OF THREE 19 NOR STEROIDS UPON LENGTHS OF CYCLES INDEXES OF OVULATION AND STEROID EXCRETION IN NORMALLY OVULATING WOMEN

Compound	Dosage (mg /day)	Number of cycles	Mean length of cycle (days)	Ovulation indexes: Basal temperature (% +)	Vaginal smear (% +)	Endometrial biopsy (% +)	Steroid output (mg /day): 17 Keto steroid	Pregnanediol
Control	—	40	27 2 ± 0 51	88	97	100	5 2 ± 0 47	3 4 ± 0 27
I	10–40	62	28 5 ± 0 68	5	4	0	4 8 ± 0 40	0 34 ± 0 066
II	10–20	34	26 7 ± 0 48	6	7	12	4 5 ± 0 47	0 30 ± 0 074
Control	—	10	25 7 ± 0 91	90	100	100	6 7 ± 0 86	3 1 ± 0 23
III	10–50	16	25 3 ± 2 24	0	0	9	8 7 ± 2 49	0 31 ± 0 38

formed corpora lutea were found (Rock *et al*, 1957) Matsumoto (1959) similarly has seen no evidence of ovulation in laparotomies of women receiving cyclical treatment with *II*

We were unable to detect any differences in potency of *I* and *II* in these studies, although their oral potency as progestins in the rabbit assay is different (see Table III) Compound *III* proved a little unusual in these studies since its use even at the highest dosages employed was marked from time to time by breakthrough bleeding, whereas in those instances where low dosages of *I* or *II* were marked by breakthrough bleeding, in

TABLE V

EFFECTS OF 17α HYDROXY 6α METHYLPROGESTERONE 17 ACETATE ALONE ON NORMALLY CYCLIC WOMEN[a]

Patient	Dose (mg)	R_x Cycle number	Ovulation index[b]		Pregnanediol (mg/day)	Cycle lengths (days)	Days of flow	Reaction
			Basal temperature	Endo metrial biopsy				
C R	1–5 to 21 2–22 to 25	1	25	–	0 3 = –	39	7	Sp
J S	1	1, 2	19 12	10, 13	2 6 = + 3 2 = +	29 25	6 6	– –
J S	4	3	15	15	1 5 = +	27	6	–
E D	4–5 to 17	1	–	n d	n d	16	n r	B T
M C	6	1, 2, 3	– – –	9 8, 14	1 2 = + 0 8 = + 1 2 = +	22 21 26	4 4 5	B T B T –
G S	10	1 2 3	+ +? n d	n d	0 2 = – 2 4 = + 4 8 = +	27 28 24	n r 3 n r	– – –

[a] Abbreviations n d = not done Sp = spotting during medication B T = breakthrough bleeding + = positive – = negative n r = no record

[b] Figures = days of ovulation

creasing the dose led to complete absence of breakthrough (Pincus *et al*, 1958a, Rock and Garcia, 1957) Furthermore, we found that the supplementation of *I*, *II*, or *III* with estrogen served to reduce markedly the incidence of breakthrough, and that of three estrogen supplements studied, diethylstilbestrol, allenolic acid methyl ether, and ethynylestradiol 3 methyl ether, the latter was most effective in reducing breakthrough to very low levels A careful study of endometrial biopsies taken at various times during medication led to the conclusion that each of the 19 nor steroids very rapidly stimulates the endometrium to progestational re

sponse but that after 7–9 days of medication the response of the endometrial glands fails, whereas the stromal cells continue to be stimulated so that by the last day of medication a predecidual state is ordinarily attained (Pincus, 1957, Pincus *et al*, 1958a, Rock *et al*, 1957, Southam, 1957)

TABLE VI

EFFECTS OF 17α HYDROXY 6α METHYLPROGESTERONE 17 ACETATE IN COMBINATION WITH ESTROGEN ON NORMALLY CYCLIC WOMEN

Patient	Dose (mg)	Estrogen (mg)	R_x Cycle number	Ovulation index[b]		Pregnanediol (mg/day)	Cycle lengths (days)	Days of flow	Reaction
				Basal temperature	Endometrial biopsy				
C R	1	ES 0 05	2 3	— —	— —	0 1 = — 0 1 = —	30 24	5 6	— —
E D	4–5 to 15 6–16 to 17	ES 0 02	2	n d	n d	n d	17	n r	B T
G G	2	EEME 0 05	1 2	10 11?	13 n d	0 6 = ± n d	25 35	4 4	+ Sp
S S	2	EEME 0 05	1 2 3	11? — —	n d	0 5 = ± 0 1 = — 0 3 = —	25 27 26	5 5 4	— — —
E P	2	EEME 0 05	1	—	12	0 4 = —	23	6	B T
E P	2–5 to 6 4–7 to 21	EEME 0 05	2	—	n d	0 3 = —	20	6	B T
E P	6	EEME 0 05	3	—	—	0 2 = —	24	n r	—
G G	10	EEME 0 05	3	11	—	0 2 = —	24	n r	—

Abbreviations n d = not done Sp = spotting during medication B T = breakthrough bleeding ES = ethynyl estradiol EEME = ethynylestradiol 3 methyl ether + = positive — = negative n r = no record

[b] Figures = days of ovulation

We have recently conducted similar studies with compounds *VI*, *IX*, and *XII* To our surprise none of these compounds gave indication of ovulation inhibition or progestational effects on the endometrium over a considerable dosage range (Rock *et al*, 1959) When, however estrogen supplementation was undertaken the effects observed with compounds *I*, *II*, and *III* became apparent Illustrative examples are given in Tables V and VI, where no significant effects except on occurrence of breakthrough

bleedıng are seen when *IX* ıs admınıstered alone, whereas when ıt ıs gıven ın adequate dose ın combınatıon with ethynylestradıol methyl ether, break through ıs ınhıbıted and pregnanedıol output ıs reduced to nonovulatory levels Experıments conducted wıth the estrogen ındıcate that ıt alone can ınhıbıt ovulatıon (Table VII) On the other hand, ıt has the effect of induc ıng prolonged perıods of menstrual flow and ıt does not exert progesta

TABLE VII

EFFECTS OF THE 3 METHYL ETHER OF ETHYNYLESTRADIOL ON NORMALLY CYCLIC WOMEN

Patıent	Dose (mg)	R_x Cycle number	Ovulatıon index[b]		Pregnane dıol (mg /day)	Cycle lengths (days)	Days of flow	Reac tıon
			Basal temper ature	Endo metrıal bıopsy				
F M	0 250	1 2, 3	– –, –	n d	0 1 = – 0 1 = – 0 2 = –	33 35 35	8 7 8	+ + –
L M	0 125	1 2 3	– 10 –	1, 12? –	0 4 = – 0 0 = – 0 1 = –	30 23 26	? 8 8	– – –
R P	0 125	1, 2 3	–, –, –	– – –	0 2 = – 0 2 = – 0 5 = ±	36 37 24	5 6 6	– – –
E S	0 125	1, 2 3	–, 8? –	–, n d	0 2 = – 0 6 = ± 0 1 = –	35 23 37	7 6 4	– + +
J B	0 05	2 3	17, 39	– –	0 1 = – 0 1 = –	32 55	6 5	– ?

[a] Abbrevıatıons n d = not done + = posıtıve – = negatıve
[b] Fıgures = days of ovulatıon

tıonal effects on the endometrıum When ıt ıs used ın combınatıon with a progestın the perıods of flow are of much shorter length (Table VI), and characterıstıc endometrıal progestatıonal effects are observed

We are thus confronted wıth an ınterestıng endocrıne puzzle Certaın compounds which are extremely potent progestıns by anımal test (e g *IX* and *XII*) appear to be relatıvely ıneffectıve ın women unless supple mented by estrogen On the other hand, compounds of much lower potency by anımal test (e g *I* and *II*) are quite actıve There ıs some evıdence that the latter may be converted to estrogens *ın vıvo* (Paulsen *et al*, 1958), and

II does have a limited intrinsic estrogenic action The inference appears inescapable that the synthetic progestins (and perhaps also progesterone itself) can exert progestational effects on the endometrium only in the presence of adequate estrogen

When it comes to a consideration of ovulation inhibition, a similar situation appears to obtain In the rabbit the more potent synthetic progestins give every indication of parallel ovulation inhibiting potency, but they are certainly ineffective in women over the expected dosage range Their potency *may* be increased by estrogen supplementation, but since the estrogen studied thus far is itself ovulation inhibiting, the role of the progestins is not calculable

V Contraceptive Trials

It became apparent from the foregoing clinical studies that contraception would be achieved using the standard regime through the simple act of ovulation inhibition To have this accompanied by menstrual cycles of normal length, supplementation with a limited amount of estrogen appeared requisite Accordingly, 10 mg tablets of compound *II* were prepared which ordinarily contained from 0 15 to 0 23 mg of ethynylestradiol methyl ether, and in April, 1956, contraceptive trials were initiated in San Juan, Puerto Rico The initiation of these trials and the methods employed have been described by Rice Wray (1957) The norethynodrel estrogen combination called Enovid was distributed in vials containing twenty of the 10 mg tablets Each subject was visited once a month at about the time of the expected menstruation and given a new supply of tablets At the time of the visit various details were obtained concerning the time of onset of menstruation, the nature of the menstrual flow, the occurrence of any reactions that might be attributable to the medication, the number of days of tablet taking that were missed, and (after some months of study) the frequency of sexual intercourse Each subject was told that if any unusual events occurred during the course of medication, communication to the physician in charge was to be made at once

Accounts of the progress of the San Juan trials have been published at intervals (Pincus 1957, 1958, 1959, Pincus *et al*, 1958a, b, Rice Wray, 1957) In April, 1957, a second project was established by Dr Clarence Gamble in a slum area outside of Humacao (R), Puerto Rico A third project was instituted among the maternity clinic patients at the Ryder Memorial Hospital in Humacao (P), and in December, 1957, a fourth project was instituted by an *ad hoc* committee of physicians in Port au Prince, Haiti An account of the data of all four projects summarized to November 1958, was presented to the Sixth International Planned Parenthood Conference in New Delhi in February, 1959 (Pincus *et al*, 1959)

In Table VIII are presented the data on the effects of the medication on the fertility of the subjects. The contraceptive effect is obvious. Inspection of the data indicated that the occurrence of pregnancy was a function of the number of days of tablet-taking that were missed in any cycle. This is clear from the data of Table IX. The conclusion is that faithful daily tablet-taking according to the prescribed regime gives a 99.8% chance of protection against conception.

TABLE VIII

THE FERTILITY OF WOMEN TAKING ENOVID IN FOUR PROJECTS IN THE WEST INDIES

Number of subjects to Nov. 1958	830
Number of active cases	519
Number of cycles of medication	8133
Number of woman years	635
Mean pregnancy rate before medication	61.2
Mean pregnancy rate during medication	2.7
Percentage reduction in pregnancies	96

* Per 100 woman years

TABLE IX

PREGNANCIES ACCORDING TO NUMBER OF TABLETS MISSED

Number of tablets missed	Number pregnant	Rate per 100 woman years
0	17	0.2
1–5	5	7.7
6–19	11	43.3

The complicating events attendant upon the contraceptive use of Enovid were: (1) menstruation during the period of medication, (2) amenorrhea following cessation of medication, and (3) reactions attributed to the medication, such as headache, gastralgia, nausea, dizziness, vomiting (cf. Tyler and Olson, 1959).

The menstruation during medication occurred chiefly because of missed tablet-taking. This is evident from the data of Table X, showing only 1.8% of short cycles in the 6931 cycles in which allegedly no tablet taking was missed, in contrast to the much higher frequencies in cycles where tablet-taking was omitted. Assuming correct reporting, then, breakthrough bleeding occurred once in every 55 cycles of medication where faithful tablet-taking was practised. Analysis of the reported frequency of premature menstruation disclosed that in the first cycle of medication the

highest incidence occurred, varying from 4.8% to 11.0% from project to project and descending to a frequency of about 1 in 60 by the fourth medication cycle, with a rather constant frequency thereafter

Amenorrhea following cessation of medication occurred in about 0.7% of the cycles Regular menstruation could usually be re established by a 20 day course of medication Now we routinely request reinstitution of medication by the tenth day after the last tablet taking if withdrawal flow fails to occur Except in cases of true pregnancy, cyclic periodicity is quickly re established

Reactions were reported in 10.2% of the medication cycles Here again the highest frequency of occurrence was in the first cycle of medication, ranging in the various projects from 17% to 38% with a descent to much lower levels (1.0% to 9.7%) by the sixth medication cycle and a characteristic constant frequency thereafter In a double blind study involving

TABLE X

Frequency of Cycles Less Than 21 Days in Length

Number of tablets missed	Number of cycles	Per cent of total cycles	Per cent of short cycles
0	6931	85.2	1.8
1–5	871	10.7	22.5
6–19	331	4.1	25.7

placebo administration versus true medication, no significant difference in the frequency of occurrence of alleged reaction was found, and in a group of women to whom no admonition concerning possible reaction occurrence was given, the frequency was extremely low On the basis of the data of these experiments, it is deducible that at least 75% of the reported reactions are psychogenic This is confirmed by the finding, in a double blind experiment in which subjects complaining of reactions were given either an antacid or a placebo, that 90% of those taking the antacid and 65% of those taking the placebo reported relief of symptoms (Pincus, 1959)

Since a proportion of those reporting side effects were probably exhibiting a physiological reaction to the medication, and since report has been made of transient jaundice in some patients taking large doses of compounds *III* and *IV* (Dunning, 1958, Seeley 1958), two liver function tests (thymol turbidity and cephalin flocculation) were conducted with 148 of the 519 active cases in October 1958 One case gave values outside the normal range in the cephalin flocculation test, but this frequency is about that to be expected in unmedicated women in the community

No evidence of medication induced pathology in the reproductive t

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TABLE X

FREQUENCY OF CYCLES LESS THAN 24 DAYS IN LENGTH

Number of tablets missed	Number of cycles	Per cent of total cycles	Per cent of short cycles
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No evidence of medication induced pathology in the reproductive tract

was found on pelvic examination, in endometrial biopsies, or in vaginal smears taken from time to time during the course of medication over a 30 month period

An analysis of the subsequent reproductive history of eighty six women withdrawing from the San Juan project demonstrated a prompt return of fertility to normal levels in those not using other forms of contraception

In field trials conducted in Los Angeles, Tyler and Olson (1959) have found a significant contraceptive effect of *I* and *II*, and have observed the various menstrual cycle phenomena seen in the experiments in our West Indies projects

A safe method of contraception is indicated by the foregoing determinations This is not too surprising since essentially physiological dosages of progestin and estrogen are administered in cyclical fashion, and the deviation from normal menstrual pattern involves (1) a type of early uterine pseudopregnancy such as might occur in women ovulating on day 5 of the cycle, and (2) a failure to ovulate Tests of pituitary gonadotropic function indicate partial suppression of gonadotropin production (Pincus, 1957, Heller, 1957)

VI Concluding Remarks

The account presented here has been concerned rather narrowly with recent studies of oral progestational agents and their significance for fertility control Many, if not all, of the compounds recently studied have rather diverse biological and clinical effects Thus *III* and *IV* are weak androgens and potent protein anabolic agents (Drill, 1959, Drill and Riegel, 1958), *X* is a weak glucocorticoid (Fried *et al* , 1958) Antiestrogenic and antiandrogenic activities have been obtained with several progestins (Drill, 1959) Among those subjected to adequate clinical trial, notable control of amenorrhea, oligomenorrhea, polymenorrhea, and dysmenorrhea has been reported (Rakoff, 1959, Southam, 1958, Weinberg, 1957) A remarkable control of endometriosis in women has been obtained, especially with *II* (Kistner, 1958), and the use of progestational agents in the treatment of threatened and habitual abortion has been the subject of numerous studies (e g Abramson, 1958, Rakoff, 1959, Reifenstein, 1958) An additional number of clinical effects have been claimed, ranging from the induction of fertility in anovulatory and other sterile women following the withdrawal of use (Rakoff, 1959, Roland, 1957) to the therapeutic treatment of osteoporosis (Nowakowski and Parada, 1958) and postsurgical convalescence (Peden *et al* , 1957)

The clinical effects are doubtless reflections of the relative predominance of certain biological activities in the special molecular configurations of individual substances We are far from an understanding of the relation

ship of structure to function in these various steroids Certain simple findings have been made, e g the lengthening of the α alkyl side chain at carbon 17 eventually leads to loss of activity, whereas the reduction of ring A saturation very nearly abolishes activity (Drill, 1959), but why, for example, the 17α methyl 19 nortestosterone should retain a degree of androgenic activity and the 17α propyl derivative lose it, with progestational activity increasing with the longer side chain, is not explained Furthermore the relationship of the metabolic fate of these compounds to their biological activity has not been examined Undoubtedly, direct studies of biochemical activity at target organs with this remarkable array of structurally diverse substances should be most informative It is already known, for example that local progestational action on the uterine endometrium is not exercised by *I* and *II*, but is powerfully exhibited by *III* and *VI* (Saunders and Drill, 1958), suggesting that in parenteral administration activity is by metabolites of *I* and *II* These metabolites may be considered simply as additional progestins probably not yet identified But what structural configurations are demanded? And why is *VI* forty times as active as *III* in the intrauterine assay?

If these and other biological questions that may be raised remain unanswered, we are certainly in default of explanations for the clinical effects Why, for example, do we see continued potent action on the human endometrial stroma accompanied by a regression in the glands? What chemical changes are induced in cervical mucus? What indeed is the mechanism of the ovulation inhibition?

Finally, considering the contraceptive effect so clearly established, what are the implications for the future? We already know that a remarkably rapid recovery of activity occurs in long time inhibited ovaries as attested by the demonstration of ovulation following cessation of medication after twenty or more months Indeed during medication the basic ovogenetic processes appear to continue unhindered (Rock and Garcia, 1959), only the formation of large follicles and corpora lutea is inhibited This also occurs with long term administration of Enovid to the rat (Pincus and Merrill, 1959) After nearly three years of field study we have seen no adverse effects of this state of ovarian quiescence

Long term administration of Enovid has had no detectable effect on extrareproductive systems, e g blood (Pincus 1959, Pincus *et al*, 1958b) We have conducted urine examinations of most of the West Indies subjects and have observed no indications of pathology Some symptoms of gastric distress may be due to direct effects of medication (presumably the estrogen used) on stomach and gut contractility, but as we have shown these tend to diminish to a low level of incidence and do not appear to be associated with detectable abnormalities An improvement in general well being

and a clear weight increase with time have, indeed, been observed in our West Indies subjects (Pincus *et al*, 1959)

A very consistent uterine response to the oral contraceptive has been seen in cycle after cycle (Pincus, 1957, Pincus *et al*, 1958b) This appears to be no different in early- or late treatment cycles With faithful use of the medication, a succession of cycles of normal length occurs to such an extent that an average tendency for more frequent than normal regularity is seen (Pincus, 1957, Pincus *et al*, 1959) Nonetheless, a more detailed study of reproductive tract processes in long term treatment would be indubitably desirable With modern analytic micromethods, aspects of endometrial and cervical mucus biochemistry could be profitably pursued Thus we have hopes of studying the effects of progestin on the carbonic anhydrase content of human endometrial biopsies Possibilities for studies of changes in carbohydrate concentrations, the fate of various enzymatic constituents, etc, exist And these are also studies basic to reproductive tract physiology in general The newer progestins offer us further variants brought to bear on the basic processes

Finally, a word concerning future possibilities of fertility control These have been ably analyzed by Nelson (1956) It suffices here to say that several alternative methods should be available There is no single method that will satisfy all men or women, however irrelevant their reasoning and reactions Any method involving female reproductive tract phenomena should allow for regular normal menstrual cycles And naturally the physiological cost should be minimal

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